Effects of pelvic 3D-CRT versus IMRT radiation therapy on circulating pro-inflammatory cytokines in high risk Prostate Cancer patients

Georgia Douvi, MD

Submitted in partial fulfillment of the requirements for the degree

Master of Science in Applied Health Sciences

(Kinesiology)

Faculty of Applied Health Sciences

Brock University

St. Catharines, ON

Abstract

This study examined the effects of pelvic radiation therapy on the levels of circulating pro-inflammatory cytokines (cPIC) in high-risk prostate cancer patients, who received pelvic radiation therapy delivered either by 3-dimensional radiation therapy pelvic (3D-CRT) or intensity-modulated radiation therapy (IMRT). Subjects included 90 patients who had not previously received treatment for their prostate cancer, and who were planned to receive androgen deprivation therapy for three years, plus concurrent pelvic and prostate boost radiation therapy. Blood samples were drawn at least 3 months into androgen deprivation therapy, before initiation of pelvic 3D-CRT or IMRT (baseline), and on days 5 and 25 of radiation treatment. Samples were analyzed for TNF-α, INF-γ, IL-4, IL-6, IL-8 and IL-10. There were no significant differences between treatment groups for any of the cytokines at any time point. When the two treatment groups were combined into a single group, a significant time/dose effect was observed for IL-4 and INF-γ, which both significantly decreased from baseline to day 25, but the effect size of this change was small (0.30 and 0.24, respectively). There was no significant time effect for the other cytokines. These results suggest that in patients with high risk prostate cancer, receiving treatment with androgen deprivation therapy and pelvic radiation therapy, cPIC are not significantly altered in response to radiation therapy compared to baseline. The small but significant changes in IL-4 and INF-γ over time suggest a potential immunomodulating effect of radiation therapy. Further studies are needed to determine the potential of cPIC as biomarkers of radiation therapy toxicity.

ACKNOWLEDGEMENTS

When I moved to Canada, I did not know what the future would hold for me. The opportunity to become a Master's student at Brock University presented a great challenge and an exciting new direction for me. There are many people I would like to thank who helped me to complete this thesis and supported me in this phase of my life.

I would like to begin my giving my sincerest and most heartfelt thanks to my supervisor, Dr. Nota Klentrou for the mentorship, encouragement, and guidance that enabled me to finish this thesis. Words cannot express how much I appreciate her time, her continuous support and her patience throughout these years. Her enthusiasm for teaching and motivating students, as well as her always positive attitude, is exactly what describes her as a person.

I also express my sincerest gratitude to Dr. Theos Tsakiridis for his valuable guidance in this journey. He trusted in me by giving me the opportunity to pursue a Master's degree in the field of oncology, knowing that my prior background in this field would help with making a good contribution to this research. I am very thankful for his support and his readiness to share his immense knowledge with me throughout the research and writing process. Without him I couldn't complete this work. A great thanks also goes to Dr. Michael Plyley who helped me immensely to complete this thesis with his extremely insightful advice, attention to detail, and valuable comments and suggestions. His kind personality and love for the students is the definition of a good teacher.

Many thanks go to the Oncology research and medical team at the Juravinski Cancer Centre who designed and carried out the clinical trial of pelvic 3D-CRT vs. IMRT and made possible the collection of biospecimens (blood samples), which permitted the study described here. I want to express my special thanks to Dr. Julie Arsenault, the Junior Principal Investigator of the clinical trial for her kind support. Her prompt and detailed responses to every question and request that I had were a huge help during this process. I would also want to thank Dr. Naghmeh Isfahanian for the time we spent together solving problems and working on many aspects of this clinical trial. Special thanks of course, goes to Dr. Ian Dayes, Senior Principal Investigator of this clinical trial.

I would also like to thank my lab mates for their valuable contributions to the successful completion of the present thesis. In particular, my sincere thanks go to Nigel Kurgan, a PhD Student at Brock University, for helping me with performing the cytokine analysis. Also, my lab mate and my best friend, Rozalia Kouvelioti, also a PhD student at Brock University, for her help on the statistical analysis of this study and for the good times had together.

The foundation of who I am can only be traced back to my parents. Their constant support, positive encouragement, and strong values helped me to become the person I am

today. I thank them for their unconditional love, motivation, and countless sacrifices. I own everything to them and thank them from the bottom of my heart.

Last but not least, I would like to express my most sincere appreciation to my husband Tom, for trusting me in achieving this endeavour and for all his endless love, moral support, patience and continuous motivation. Without him I wouldn't even been able start this journey. Most importantly, I am deeply grateful to the most treasured person in my life: my son Philippos. You were the reason to stand up every time I was down during the difficult days through this process. You have made me stronger, better and more fulfilled than I could have ever imagined. I love you more than you can imagine. You are my everything.

The clinical trial was funded by Sanofi Canada and the Juravinski Cancer Center Foundation to Drs Ian Dayes, Julie Arsenault and Himu Lukka, principal investigators. The biospecimen collection was funded by an AbbVie – CARO Uro-Oncologic Radiation (ACURA) award from the Canadian Association of Radiation Oncology to Dr. Theos Tsakiridis, and the cytokine analysis was funded by a Brock University Advancement Grant.

I dedicate this thesis to my parents, to my husband Tom and my precious son Philippos.

Table of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

IMRT	Intensity-modulated radiation therapy
3D-CRT	3-Dimensional conformal radiation therapy
cPIC	Circulating pro-inflammatory cytokines
PSA	Prostate specific antigen
TNM	Tumor Stage
IL	Interleukin
TNF-α	Tumor Necrosis Factor-α
TGF-β	Transforming growth factor-β

CHAPTER 1: INTRODUCTION

1.1 Background and Rationale

Prostate cancer is the most common cancer (excluding non-melanoma skin cancers) and the 3rd leading cause of death Canadian men (1). It is estimated that 21,300 men will be diagnosed with prostate cancer in 2017, which represents 21% of all new cancer cases in men. Of these men, it is predicted that 4,100 will die from prostate cancer, amounting to 10% of all cancer-related deaths (1).

Prostate cancer treatment options depend on several factors, such the extent of the disease, the stage of the cancer, the patient's age, operability, co-morbidities, and overall health. For very low-risk prostate cancer patients, recommended treatment may involve active surveillance and regular follow-ups. In early stage, prostate cancer is curable with prostate excision surgery (radical prostatectomy) or local, prostate alone, radiation therapy, while adjuvant or salvage radiation therapy can be used following radical prostatectomy for residual disease or disease recurrence, respectively (2,3). Although surgery can be used in locally invasive, high risk prostate cancer, standard therapy for this disease involves prostate as well as pelvic lymph node RT in combination with androgen deprivation therapy delivered over 18-36 months (2,3).

1.2 The Need for Biomarkers

The risk for prostate cancer metastasis to distant sites and therefore, its response to local therapies, such as surgery and radiation therapy, is determined by the disease stage that is typically defined by the prostate specific antigen (PSA), clinical stage (TNM), and the pathologic Gleason score. The pre- and post-treatment value of prostate

specific antigen are used to predict survival rates and responses to radiation therapy (4). However, it is difficult to individualize treatments based on current pre-treatment factors, and there is no means to predict toxicity once radiation therapy has begun. Therefore, new, predictive biomarkers are needed in radiation oncology to predict toxicity and customize individual patients' treatment (5,6).

Over the past two decades, circulating pro-inflammatory cytokines (cPIC) have been investigated by researchers as a predictive biomarker of acute and late toxicity due to radiation therapy (6). The production and release of cytokines can be affected by the cancer itself, and treatments, such as radiation therapy, can cause inflammation, with subsequent release of cytokines in various tissues. High levels of circulating pro-inflammatory cytokines have been associated with the development of toxicity, which can occur in irradiated normal tissues during treatment (7,8). Several studies have shown higher levels of circulating pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α), following radiation therapy. The prognostic relevance of circulating pro-inflammatory cytokines in different forms of cancer (soft-tissue and adult bone sarcoma, breast, pancreatic, gastric, kidney, lung cancer head and neck) are of significance to clinicians (7,8). However, few studies have examined the role of circulating pro-inflammatory cytokines in predicting radiation induced toxicity for prostate cancer patients (7)

Therefore, the aims of the present study were: (a) to examine the effects of radiation therapy on the levels of circulating pro-inflammatory cytokines in high risk cancer patients that receive pelvic radiation therapy, and (b) to assess the viability of using the levels of circulating pro-inflammatory cytokines as potential biomarkers of

tissue toxicity. To this end, the levels of circulating pro-inflammatory cytokines were investigated in patients who participated in a randomized clinical trial that examined the impact of delivering pelvis radiation therapy via two protocols, intensity-modulated radiation therapy (IMRT), which was compared to 3-dimensional conformal radiation therapy (3D-CRT).

This project was part of a larger clinical trial, which examined the potential of a modern radiation therapy technique (IMRT), aimed at reducing normal tissue toxicity as a means of improving patient quality of life.

CHAPTER 2: LITERATURE REVIEW

2.1 Prostate Cancer

The prostate gland is an ovoid shaped structure, composed of fibrous, granular and muscular elements. It is located in the pelvis, adjacent to the rectum, bladder, dorsal and periprostatic venous complexes, pelvic sidewall musculature, the pelvic plexus and cavernous nerves. The main function of the prostate is the production of seminal fluid, which protects and nourishes the sperm after ejaculation (2).

Prostate cancer refers to the growth of abnormal cancer cells in the prostate. The majority of prostate cancers start in the prostate gland, and are referred to as adenocarcinoma (>95%). There are other types of cancer cells that start in the prostate, but adenocarcinoma is the most common (2,3).

2.2 Screening and Diagnosis for Prostate Cancer

The two tests commonly used to screen for prostate cancer are: the prostate specific antigen (PSA) blood test and the digital rectal exam. PSA is a protein produced by both normal and cancerous prostate cells that is released into the blood. High levels of prostate specific antigen are thought to be indicative of prostate cancer or non-cancerous inflammatory conditions.

There is no specific normal or abnormal level of PSA in the blood. In the past, PSA levels of 4.0 ng/mL and lower were considered as normal. However, baseline PSA values vary widely amongst individuals, and some men with PSA levels below 4.0 ng/mL have prostate cancer, while men with higher levels have benign prostatic hyperplasia and

do not have prostate cancer. In addition, hormonal factors, inflammation, and even mechanical pressure on the prostate can cause the PSA level to fluctuate (2,3).

2.2.1 Gleason score

Prostate cancer tumours are graded using the Gleason scoring system, which evaluates architectural details of individual cancer glands, and describes five distinct growth patterns from Gleason 1 (well differentiated) to Gleason 5 (poorly differentiated). The two commonest growth patterns (primary and secondary) seen are submitted to give a final Gleason score(GS) ranging from 2 (1+1) to 10 (5+5) (Figure 1).

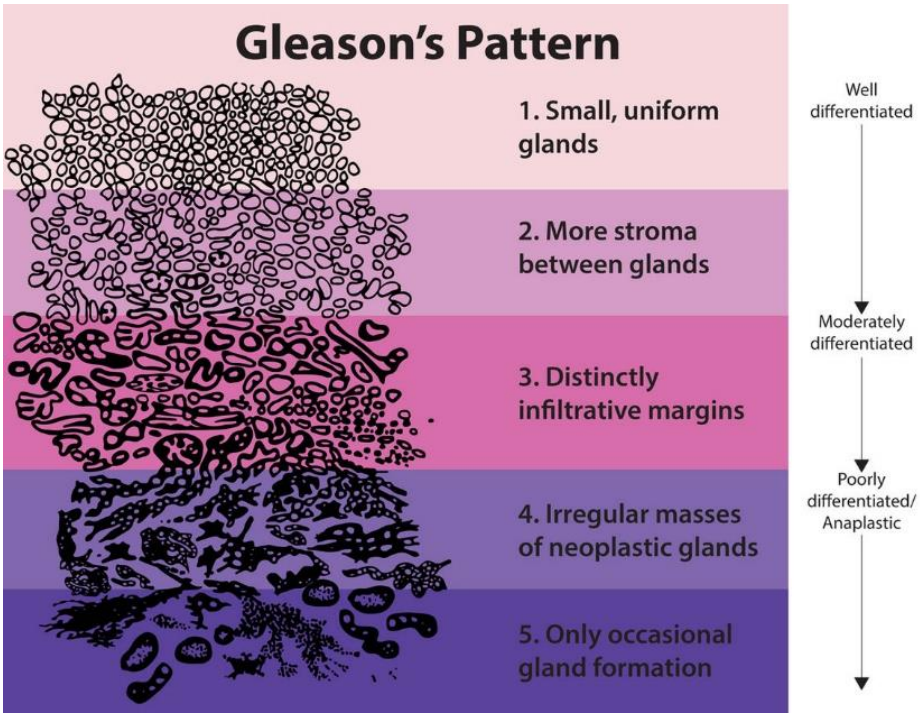


Figure 1. Gleason's Pattern**.**

2.2.2 TNM staging system

Staging is a way of describing or classifying a cancer based on the extent of cancer in the body. The most common staging system for prostate cancer is the TNM system shown in Figure 2. TNM staging describes the size and extent of the primary tumour, the number and location of any regional lymph nodes that have cancer cells in them, and whether the cancer has spread or metastasized to another part of the body. For clinical staging prior to treatment, the extent of the tumour is described on the basis of clinical tests. Pathological staging describes the true extent of cancer in the prostate after it has been removed surgically (see Figure 2).

TABLE 1: 2010 TNM staging system of prostate cancer

Localized disease	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in ≤ 5% of resected tissue
T1b	Tumor incidental histologic finding in > 5% of resected tissue
T1c	Tumor identified by needle biopsy (eg, because of elevated PSA level)
T2	Tumor confined within prostate
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
Local extension	
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Bladder invasion, fixed to pelvic side wall, or invasion of adjacent structures
Metastatic disease	
N1	Positive regional lymph nodes
M1	Distant metastasis

From Edge SB, Byrd DR, Compton CC, et al (eds): AJCC Cancer Staging Manual, 7th ed. New York, Springer, 2010.

Figure 2. TNM Staging system for prostate cancer (AJCC Staging Manual, 2010, 7th Edition)

The stage of the cancer refers to the extent of disease based on tumour location and size, and whether the cancer has spread outside the prostate gland to surrounding organs. Stages I and II refer to early-stage disease that is confined to the prostate. Stage III refers to locally advanced disease that has spread outside the prostate gland. Stage IV refers to cancer that has metastasized and possibly spread to the lymph nodes and other organs in the body. See figure 3.

TABLE 2: TNM staging system of prostate cancer, 2010 updates[a]

Anatomic Stage/Prognostic Groups

GROUP	T	N	M	PSA	Gleason
Stage I	T1a–c	N0	M0	PSA < 10	Gleason ≤ 6
	T2a	N0	M0	PSA < 10	Gleason ≤ 6
	T1–2a	N0	M0	PSA X	Gleason X
Stage IIA	T1a–c	N0	M0	PSA < 20	Gleason 7
	T1a–c	N0	M0	PSA ≥ 10 < 20	Gleason ≤ 6
	T2a	N0	M0	PSA < 20	Gleason ≤ 7
	T2b	N0	M0	PSA < 20	Gleason ≤ 7
	T2b	N0	M0	PSA X	Gleason X
Stage IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1–2	N0	M0	PSA ≥ 20	Any Gleason
	T1–2	N0	M0	Any PSA	Gleason ≥ 8
Stage III	T3a–b	N0	M0	Any PSA	Any Gleason
Stage IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

From Edge SB, Byrd DR, Compton CC, et al (eds): AJCC Cancer Staging Manual, 7th ed. New York, Springer, 2010.
[a]When either PSA or Gleason is not available, grouping should be determined by T stage and/or either PSA or Gleason as available.

Figure 3. Anatomic stage/prognostic groups of Prostate cancer (AJCC Cancer Staging Manual, 2010, 7th Edition)

2.3 Role of Radiation Therapy

Radiation therapy plays an important role in treating many forms of cancer (along with surgery and chemotherapy). Radiation therapy uses high energy ionizing rays (x-rays or gamma rays) to destroy cancer cells by damaging the DNA within the cancer cells, thereby destroying the cells' ability to divide and proliferate. Normal cells also sustain DNA damage, but they are able arrest their cell cycle in an organized fashion and repair their DNA (9).

During radiation treatment, the dose of the radiation is escalated in a planned fashion to improve control of the radiation dose to the cancerous cells, and minimizing the dose to neighbouring cells (10,11). While radiation therapy dose escalation has improved local control of tumour growth, higher doses of radiation can lead to an increased toxicity to normal organs at risk, such as early and late gastrointestinal and genitourinary toxicity (11,12).

Starting in late 1990'S, 3-dimensional conformal radiation therapy (3D-CRT) began to replace standard field radiation due to its benefits in focusing the radiation to the cancerous cells, thereby, reducing acute and late side effects to normal organs at risk. However, side effects remained, especially when employing high doses (13).

Since the survival rate for most prostate cancer patients is generally greater than 10 years, choosing a radiation therapy technique that minimizes radiation dose to surrounding healthy organs is important in improving patients' quality of life (10,11). The challenge of increasing the radiation dose for prostate cancer treatment, while minimizing toxicity effects, has led to the development of new radiation therapy delivery techniques, one of which is intensity-modulated radiation therapy (IMRT) (14).

IMRT is a more modern technique that uses treatment fields with highly modulated radiation dose patterns to deliver exquisitely conformal radiation distributions. Rather than defining a fixed field shape with differential weighting, as is done in conventional treatment planning, IMRT treatment utilizes an inverse planning approach whereby the desired dose to the target and normal tissues is specified using mathematical descriptions referred as “constrains” or “objectives”, which define the principals according to which the radiation therapy plan is developed (2). IMRT for prostate radiation therapy delivers radiation from multiple angles using beams of different strengths, targeting a higher dose to the prostate and less to surrounding organs. Studies have shown that IMRT is associated with reduced long-term toxicities and excellent biochemical control outcomes compared to 3D-CRT (3).

The ability of IMRT to reduce the dose applied to nearby organs at risk, without sacrificing coverage of the target structures, and perhaps improve target coverage, is clinically appealing, and has led to the rapid adoption of this technology for the treatment of prostate and other cancers (3).

2.3.1 Different types of radiation techniques

Radiation therapy is a treatment that has had a significant role in treating localized prostate cancer. Historically, radiation treatment has passed through different phases over time with considerable improvements in quality of radiation delivery (2,3).

In modern radiation therapy, computed tomography/magnetic resonance imaging and sophisticated planning software are used to analyze and plan precise dose delivery and distribution to the target tumour and minimize radiation delivery to nearby organs at risk. This interaction between planning software and imaging has allowed the

development of more efficient conformal plans, which in turn prompted led to the development of 3D-CRT and IMRT (3).

Radiation therapy is the standard of care curative therapy for localized early and advanced prostate cancer. It may be delivered in the form of external-beam radiation therapy or brachytherapy (i.e., the insertion of radioactive seeds into the prostate gland). Only external-beam radiation therapy techniques will be discussed here (3).

Conventional external beam radiation therapy to the pelvis, with limited conformality, was the standard of radiation therapy care for many years and was typically delivered using a 4-field technique. The 4 fields (anteroposterior, posteroanterior, left lateral, and right lateral) were designed to include the prostate, the seminal vesicles, and the regional lymphatic vessels. Conventional external beam radiation therapy involved irradiation of large volumes of tissue, including the skin, small bowel, bladder, large bowel, pelvic bones, and additional areas of soft tissue, and therefore, it is not surprising that toxicity of normal tissues was problematic (2,3,4). Up to the early 2000s, external beam radiation therapy to the pelvis had limited conformality, and the radiation beam was shaped by using custom made lead blocks that were fitted on the radiation therapy unit to provide some limited conformality to the radiation beams.

3D-CRT was developed when technological advances permitted the development of the multi-leaf collimator device, which permitted the generation of radiation therapy beams that were more conformal to the target. In 3D-CRT, the radiation beam is shaped to include the 3D anatomic configuration of the prostate and any specified adjacent tissue (including the seminal vesicles and periprostatic adventitial tissues). This technique

allows more precise delivery of the radiation therapy to the target organ or organs (2,3,15).

IMRT is a relatively recent refinement of the 3D-CRT technique. IMRT uses treatment fields with highly irregular radiation intensity patterns to deliver exquisitely conformal radiation distributions. IMRT can achieve tightly conformal dose distributions through the use of non-uniform radiation beams. The intent of this form of therapy is to create highly conformal fields by treating the patient with multiple static portals or dynamically modulated fields (so-called step-and-shoot IMRT) at specific angles. Delivery of IMRT requires a computer-controlled beam shaping apparatus on the linear accelerator, known as multi-leaf collimator. The multi-leaf collimator used for IMRT consists of many small individually moving leaves that can create multiple beam shapes (3,15).

2.3.2 Role of androgen deprivation therapy on prostate cancer

Androgen deprivation therapy is the cornerstone of medical treatment for advanced prostate cancer and is used as a neoadjuvant, concurrent, and adjuvant therapy for high risk disease (16,17). The growth of the prostate gland is regulated by androgens. The primary androgen being testosterone. Generally, androgens are required for the development of prostate cancer, and changes in androgen activity over time can affect carcinogenesis and disease progression (2).

The purpose of androgen deprivation therapy is to block the production of testosterone, and it is a well-established treatment for advanced prostate cancer (17). Androgen deprivation therapy has been increasingly used in combination with radiation therapy in high risk prostate cancer patients (16,17).

There are many options of using androgen deprivation therapy, but the following are the most popular. Surgical removal of one or both of the testicles (orchiectomy) to prevent testosterone production. This is very effective in that is can reduce the level of testosterone by 90-95% but is not often used because of its permanent effect. Another option is medical castration with gonadotropin releasing hormone agonists. Long-term treatment with gonadotropin releasing hormone agonists supplant the effect of the pulsatile release of endogenous gonadotropin releasing hormone, and is thought to down-regulate its receptors in the pituitary gland, leading to castration levels of testosterone within 3 weeks (2,3).

Androgen deprivation therapy has been increasingly used in combination with radiation therapy in the treatment of intermediate to high risk prostate cancer. The current standard of care for patients diagnosed with high-risk prostate cancer confirmed by histology is the combination of neoadjuvant, concurrent and adjuvant androgen deprivation therapy (for a total of 3 years) and radical radiation therapy. Luteinizing hormone-releasing hormone agonists are long-acting analogs of the native luteinizing hormone-releasing hormone peptide and are effective at reducing serum testosterone.

Androgen deprivation therapy has been shown to improve the survival rate when combined with radiation therapy. Bolla et al. (2002) showed that long term androgen suppression with luteinizing hormone-releasing hormone agonist improves 5 year survival rate to 78%, compared to 62% with radiation therapy alone (17).

2.3.3 Current standard of care for high risk Prostate cancer

High risk prostate cancer is defined as a tumour of any stage (T1-T4) with a Gleason score of 8-10, or a PSA above 20, or a tumour determined to be stage T3 or

higher. The current standard of care for high risk prostate cancer has been the use of hormonal therapy, in a neo-adjuvant, concurrent and adjuvant setting, in combination with radiation therapy.

2.4 Radiation Therapy Toxicity in Prostate Cancer Patients

Regardless of the radiation mode used, radiation therapy for prostate cancer can cause major acute or late side effect complications to the surrounding normal organs at risk (18). Acute toxicity occurs when the side effects of radiation therapy occur less than 12 weeks from the completion of the treatment. Late toxicity occurs when the side effects occurred after 12 weeks of the completion of the treatment. Gastrointestinal and genitourinary toxicity are the most important complications and can lead to life threatening conditions. Dose escalation with 3D-CRT improves the biochemical control of prostate cancer, but it can lead to acute and late toxicity to normal organs at risk.

Several studies have suggested that IMRT reduces the radiation dose to the normal organs at risk, leading to lower rates of acute and late toxicity, even at high dose escalation (>74 Gray (Gy)) (19-21). Mamgani et al. compared the acute and gastrointestinal and genitourinary toxicity in prostate cancer patients to a total dose 78Gy delivered via 3D-CRT or IMRT techniques. The results show that IMRT reduced the toxicity without compromising the outcome in patients (11). Zelefsky et al. also showed the benefit of high-dose IMRT for patients with localized prostate cancer with respect to dose conformality relative to tumor coverage and exposure to normal organs. The authors concluded that IMRT represents the safest technique when using higher radiation doses in prostate cancer (18). Mok et al. showed that IMRT allowed for the delivery of high conformal radiation therapy to the target volume, while lowering the dose to surrounding

organs at risk, compared to 3D-CRT (20). Fenoglietto et al. concluded that the dose coverage of the planned target volume and organs at risk was better with IMRT and remained so, even when the internal volume changed (12).

In a recent meta-analysis, Yu et al. evaluated 23 eligible studies involving ration therapy with prostate cancer (13). IMRT was shown to result in a decreased grade 2-4 for both acute and late acute gastrointestinal toxicity, and late rectal bleeding compared with 3D-CRT. However, the study also showed that IMRT increased grade 2-4 tumours in terms of both acute and late genitourinary toxicity. The study concluded that generally, IMRT provides better biochemical control than 3D-CRT (13). In another recent, randomized clinical trial, Viani et al. reported that the percentage of the bladder and rectal volume receiving doses between 54 Gy to 62 Gy was statistically lower for IMRT versus 3D-CRT, and resulted in a reduced acute/late grade >2 gastrointestinal and genitourinary toxicity compared to 3D-CRT (21). The authors concluded that this study provided further support to the idea that reduced dosage to the normal organs at risk using IMRT provides clinical benefits to prostate cancer patients.

2.4.1 Biomarkers of toxicity

Understanding the biologic events associated with gastrointestinal and genitourinary toxicity in patients receiving pelvic radiation therapy can help researchers understand the mechanisms leading to the development of this toxicity. Further, identifying biomarkers that could predict the development of toxicity in patients that are being treated with radiation therapy would have significant benefits for patients, and possibly allow for individualized treatment plans. The focus of the present study was to

examine whether circulating pro-inflammatory cytokines can serve as potential markers of toxicity, prior to and during radiation therapy delivered by two different protocols.

2.5 Cytokines

Cytokines are a large group of proteins, peptides or glycoproteins that are secreted by certain cells of the immune system. They are a category of hormone-like factors that mediate and regulate immunity, inflammation, and hematopoiesis (22). Cytokines can have either pro- or anti-inflammatory and immunosuppressive activity, depending on the microenvironment. A cytokine network is shown in Figure 4 below.

Cytokines are affected by the cancer itself. The tumour microenvironment is rich in cytokines and other inflammatory mediators that influence immunosuppression, growth of cancer cells, tissue remodelling, and angiogenesis (7,23,24). Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action) (6,23).

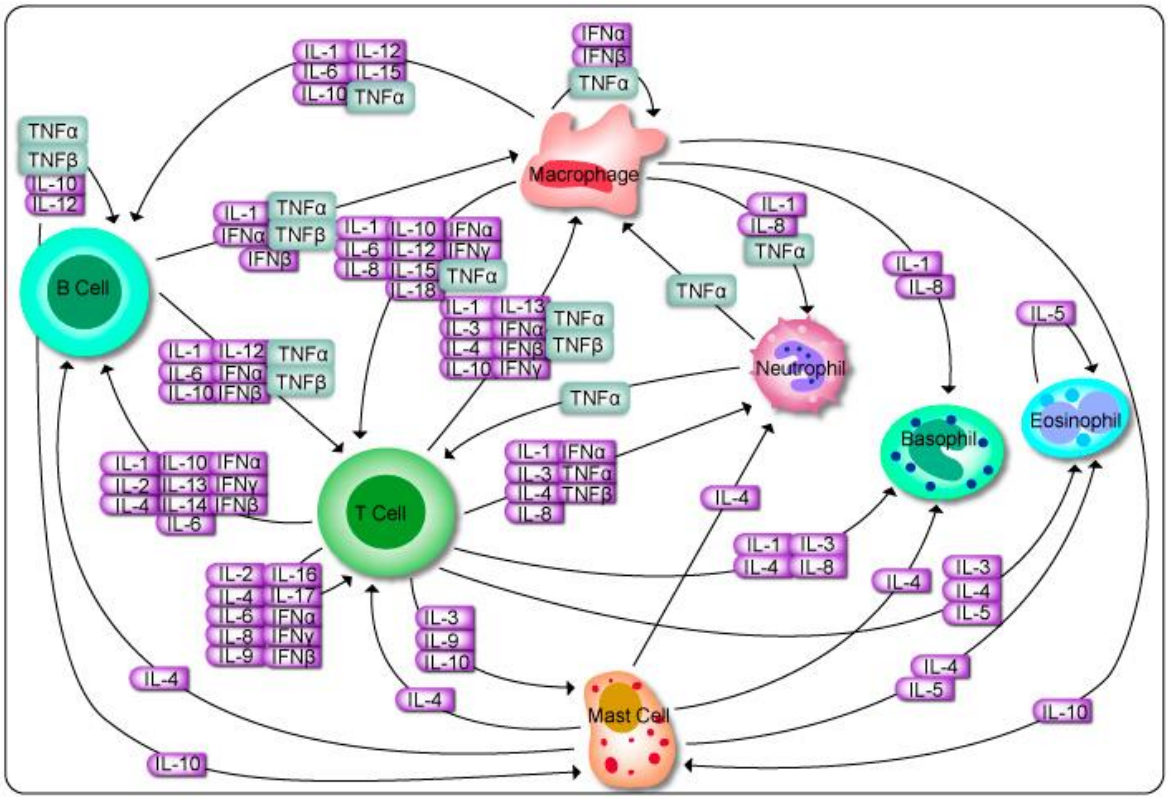


Figure 4. Cytokine network (Kyoto Encyclopedia of Genes and Genomes, www.genecopoeia.com)

2.5.1 Association of pro-inflammatory cytokines with radiation therapy in cancer

While the mechanisms responsible for late side effects of radiation therapy are largely unknown, it is believed that circulating cytokines are involved in the mediation of radiation therapy toxicity. Furthermore, there is significant interest in the potential use of circulating cytokine levels as predictive biomarkers of the side effects of radiation therapy (8,23,25). More recently, elevated pro-inflammatory and pro-fibrotic cytokines have been observed in the plasma of patients receiving radiation therapy, thereby providing additional support for the existence of a humoral response to radiation therapy (26). Abnormal levels of cytokine production induced by radiation have been suggested as being a contributing factor by multiple investigators. It has been recently shown that

ionizing radiation can increase the expression of these cytokines, and there are indications that increased levels of cytokines may be associated with morbidity (23).

Rubin et al. (25) were among the first researchers to describe the role of cytokines related to radiation toxicity. In pre-clinical and clinical lung studies, they showed that the levels of IL-1, transforming growth factor (TGF)-β, and TNF-α were increased immediately after radiation exposure, and that chronically elevated TGF-β levels were associated with an increased risk of pulmonary fibrosis.

The concept of using cytokine expression during radiation therapy as a predictive biomarker of toxicity outcomes is promising (45,47). In a study focused on thoracic radiation therapy, the circulating pro-inflammatory cytokines, IL-1a and IL-6 and the profibrotic cytokine TGF-β, were linked to the temporal development of radiation-induced inflammatory pneumonitis and pulmonary fibrosis, respectively (27). Seruga et al. list several studies describing the levels of circulating pro-inflammatory cytokines in different types of cancer (see Table 1). Significantly, higher levels of IL-6, IL-8 and IL-10 were observed in breast cancer, pancreatic cancer, soft tissue sarcoma, and adult bone sarcoma. In addition, high levels of IL-6, and IL-8 were found in head and neck cancer. In gastric cancer, IL-6 was also high, indicating a higher stage of the disease. This study did not report any studies for prostate cancer and Hodgkin lymphoma (7).

Table 1. Observational studies of levels of circulating cytokines and their prognostic significance in cancer (adopted from Seruga et al., 2008).

Type of Cancer	Number (Setting)	Reported changes in circulating levels of Cytokines	Prognostic Significance	Ref.
Soft-tissue sarcoma	145 (non-metastatic) 50 healthy controls	Significantly higher levels of IL1RA, sIL2R, IL6, IL8, IL10, TNFRI, TNFRII, TNFα , M-CSF, FGF2 and VEGF than controls	NO	19
Adult bone sarcoma	72 (non-metastatic) 50 healthy controls 22 controls with	Significantly higher levels of IL6, IL8, L10, VEGF, FGF2, M-CSF, IL1RA, TNFRI and TNFRII than healthy controls; significantly	Higher levels of IL1RA and TNFRI are an independent predictor of shorter OS	20

	benign tumors	higher levels of IL6, IL8, IL1RA, TNFRI and M-CSF than in patients with benign bone tumors		
Breast cancer	111 (non-metastatic) 36 healthy controls	Significantly higher levels of IL6 than in healthy controls	NA	21
	45 (non-metastatic and metastatic) 25 healthy controls	Significantly higher levels of IL6, IL8 and IL10 than in healthy controls. Patients with higher stages (stage III and IV) had higher levels of cytokines compared with patients with stage II	NA	209
	96 (progressive metastatic)	Significantly higher levels of IL6 in patients with higher burden of metastatic disease than those with lower burden	Higher levels of IL6 are associated with shorter OS	206
	65 (recurrent) 17 (non-recurrent)	Significantly increased levels of IL6 in people with recurrent breast cancer compared with non-recurrent breast cancer	NA	210
	77 (metastatic) 64 (non-metastatic) 27 healthy controls	Significantly higher levels of IL8 in non-metastatic and metastatic disease compared with healthy controls, and in metastatic disease as compared with non-metastatic disease	Higher levels of IL8 are an independent predictor of shorter OS in women with metastatic disease	22
Pancreatic cancer	51 (non-metastatic) 48 healthy controls	Significantly higher levels of IL6, IL8, IL10 and IL1RA than in healthy controls	High levels of IL6 are an independent predictor of shorter OS	23
Gastric cancer	155 (non-metastatic) 63 healthy controls	Higher levels of IL6 than in healthy controls Higher levels of IL6 associated with a higher stage of the disease	Higher levels of IL6 are an independent predictor of shorter OS	24
Kidney cancer	64 (non-metastatic) 12 controls with benign tumors	Significantly higher levels of IL6 and IL10 than in patients with benign disease at diagnosis and 3 months after resection of the primary tumor	NA	25
	138 (metastatic)	IL6 detectable in 70%, IL10 in 8% and VEGF in 71% of patients, respectively	Higher levels of IL6 are an independent predictor of shorter PFS and OS	207
Prostate cancer	423 (non-metastatic)	Not reported	Preoperative levels of sIL6R and TGFβ increased the accuracy of classical nomogram to predict biochemical recurrence	208
Head and neck cancer	40 (non-metastatic) 20 healthy controls	Significantly higher levels of IL6 and IL8 than in healthy controls	NA	26
	11 (non-metastatic) 12 controls with benign tumors 12 healthy controls	Significantly higher levels of IL6, IL8 and VEGF than in healthy controls and patients with laryngeal papilloma	NA	243
	58 (non-metastatic)	Significantly higher levels of IL6 and IL10 and lower levels of IL12 in patients with higher tumor and node stage of primary tumor	NA	211
	57 (non-metastatic) 40 healthy controls	Significantly higher levels of IL10 and lower levels of IL12 than in healthy controls; higher IL10 levels associated with higher tumor stage	NA	27
Hodgkin lymphoma	519 (at diagnosis)	Not reported	High levels of IL1RA and IL6 are an independent predictor of shorter EFS	244
AML, MDS	198 (at diagnosis) 48 healthy controls	Significantly increased levels of TNFα , IL1RA, IL6 and IL10 than in healthy controls	Higher levels of TNFα was associated with lower CR rate, EFS and OS (not an independent prognostic factor)	28
	54 (at diagnosis) NS healthy controls	Significantly higher levels of IL1, IL1RA, IL6, IL8 and TNFα than in healthy controls	NA	109

AML, acute myeloid leukemia; CR, complete remission; EFS, event-free survival; FGF2, fibroblast growth factor 2; IL, interleukin; IL1RA, IL1 receptor antagonist; M-CSF, macrophage-colony stimulating factor; MDS, myelodysplastic syndrome; NA, not assessed; NS, not stated; OS, overall survival; PFS, progression-free survival; sIL2R, soluble IL2R; TNF, tumor necrosis factor; TNFR, TNF receptor; VEGF, vascular endothelial growth factor.

2.5.2 Association of pro-inflammatory cytokines with radiation therapy in prostate cancer

Limited studies have been done so far to examine the levels of cytokines in patients with prostate cancer (7,44). Cytokine expression is associated with radiation-related organ damage and may be useful to help predict toxicity. Christensen et al. observed that IL-6, IFN-γ, IL-1, and IL-2 may be important cytokine biomarkers of acute gastrointestinal and genitourinary toxicity (28). In contrast, a relatively recent study by Dirksen et al. suggest that there is no strong correlation between radiation therapy induced symptoms (urinary irritative, bowel problems, etc.) and cytokine levels (29). Patients undergoing 3D-CRT for prostate cancer had an increase in circulating IL6 levels within 15 days of radiation therapy, which returned to baseline levels soon after. In contrast, IL-2, IL-4, IL-5, TNF-α, macrophage inflammatory protein 1-alpha, and leukemia inhibitory factor levels were unchanged with radiation therapy (30). Kovacs et al. found elevated circulating cytokines ($P<0.05$), even prior to radiation therapy, which increased even higher during radiation therapy (26). This work forms the basis for the development of a prospective radiation therapy trial in which IL-6, IFN-γ, IL-1, and IL-2 are assessed as potential biomarkers of radiation toxicity, which could ultimately alter patient management during radiation therapy.

2.5.3 Impact of androgen deprivation therapy on pro-inflammatory cytokines

According to the literature, androgens tend to be immunosuppressive in nature (30). Studies have examined the role of androgen deprivation therapy on circulating

cytokine levels. Jonke et al. examined the levels of circulating cytokines in two patient groups: one receiving radiation therapy alone, and the other receiving radiation therapy + androgen deprivation therapy. The results showed that while the cytokine response was similar between both groups, the magnitude of the cytokines levels was noticeably different between the two groups, with IL-1β and IL-6 being higher in patients receiving radiation therapy + androgen deprivation therapy compared to radiation therapy alone, while TGF-β was lower in patients receiving radiation therapy + androgen deprivation therapy compared to radiation therapy alone. The authors further mentioned that androgen deprivation therapy may shift the ratio of pro-inflammatory and profibrotic cytokines toward a more immune-stimulatory state (30).

Maggio et al. examined 3 groups of men: 1) 20 men with prostate cancer undergoing androgen deprivation therapy for at least 12 months prior to the onset of the study (androgen deprivation therapy group); 2) 18 age-matched men with non-metastatic prostate cancer who had undergone local surgery and/or radiation therapy, but had not yet received androgen deprivation therapy and were eugonadal (non-androgen deprivation therapy group); and 3) 20 age-matched healthy eugonadal men (control group). They found that no significant differences in serum levels of pro-inflammatory or anti-inflammatory cytokines between the 3 groups. These data suggest that men with prostate cancer undergoing long-term androgen deprivation therapy do not have elevated levels of pro-inflammatory cytokines compared to age and disease matched controls (32). Furthermore, a recent study by Tanji et al. showed that androgen deprivation therapy, in combination with radiation therapy, influenced certain pro-inflammatory cytokine levels in prostate cancer patients as evidenced by significantly increased levels of epidermal

growth factor (EGF), granulocyte-colony stimulating factor (G-CSF), and IFN-γ during radiation therapy (33). Both these studies report that the immunological responses to androgen deprivation therapy alone, or in combination with radiation therapy, are not well understood, and that more research is needed in this area.

CHAPTER 3: OBJECTIVES AND HYPOTHESIS

3.1 Overall purpose

There is a need to identify potential blood borne biomarkers in pre-treatment or early treatment blood samples that can reliably predict radiation therapy induced gastrointestinal and genitourinary toxicity in high risk prostate cancer patients. Circulating pro-inflammatory cytokines levels in the blood show some promise as potential clinical biomarkers. If specific circulating cytokine levels prior to, or during, radiation therapy are found to be statistically associated with gastrointestinal and genitourinary toxicity, then patients could be identified early, targeted for specific intervention, including modification of radiation dose, and followed closely post-treatment.

One way to investigate this issue further is to measure cytokine levels in high risk prostate cancer patients who have received either 3D-CRT or IMRT. Thus, the overall purpose of the present study was to compare cytokine levels in the pre-treatment and early treatment blood samples of patients receiving IMRT vs 3D-CRT pelvic radiation therapy for high risk prostate cancer.

3.1.1 Specific Objectives

The specific objectives of this project were:

i) to examine how pelvic radiation therapy for high risk prostate cancer patients influences the levels of circulating pro-inflammatory cytokines;

ii) to determine whether the circulating pro-inflammatory cytokines response to pelvic radiation therapy differs amongst patients treated with 3D-CRT vs IMRT.

3.1.2 Hypothesis

The central hypothesis is that there will be statistically significant higher levels of circulating pro-inflammatory cytokines in patients that received 3D-CRT compared to IMRT for the same radiation dose to the pelvis.

3.2 Long-term Goals

The current project is part of a larger clinical trial, the long-term goal of this research is to determine: i) whether circulating pro-inflammatory cytokines levels are closely associated with radiation therapy dose delivered to organs at risk and patient quality of life, and ii) whether circulating pro-inflammatory cytokines levels can be used as predictive biomarkers of radiation therapy toxicity during pelvic radiation therapy for prostate cancer.

To achieve this goal, it is necessary to examine cytokine levels over time, i.e., prior to initiating treatment, and over the course of early radiation therapy, and correlate these results with dosimetry parameters, toxicity assessments, and measures of patient quality of life.

The results of the present study are important in the treatment of cancer as the results could lead to the development of individually tailored radiation therapy treatment for prostate cancer. The goal would be to develop treatment to provides improved local control and the preservation of the patients' quality of life.

CHAPTER 4: METHODS

4.1 Study Population

The procedures involved in this study received ethics approval from both the Juravinski Cancer Center and Brock University Research Ethics Boards. One hundred and four patients, who had not previously received treatment for their prostate cancer, were accrued to the larger clinical trial. Eligibility for the clinical trial was based on a diagnosis of high-risk adenocarcinoma of the prostate, confirmed by histology (the UICC-TNM staging system was used) and defined as tumours with one or more of the following criteria: a tumour of stage T1-T4 with a Gleason score of 8-10, or a PSA above 20, or a tumour determined to be stage T3 or higher. Participating patients have not previously received treatment for their prostate cancer. Patients also received hormone therapy (neoadjuvant, concurrent and adjuvant for 2 to 3 years).

Of all the 104 patients who agreed to participate in the study and provided informed consent, only 90 patients agreed to have their blood samples collected (the other 14 declined this portion of the trial). From the 90 patients involved in the present study, blood samples from 75 patients – 35 treated with 3D-CRT (mean age 70 ± 12.9), and 40 patients treated with IMRT (mean age 68 ± 19.0), were collected and analyzed for cytokine levels over the course of their radiation treatment. The remaining samples (n=15) were not analyzed due to technical and storage issues (see Figure 5).

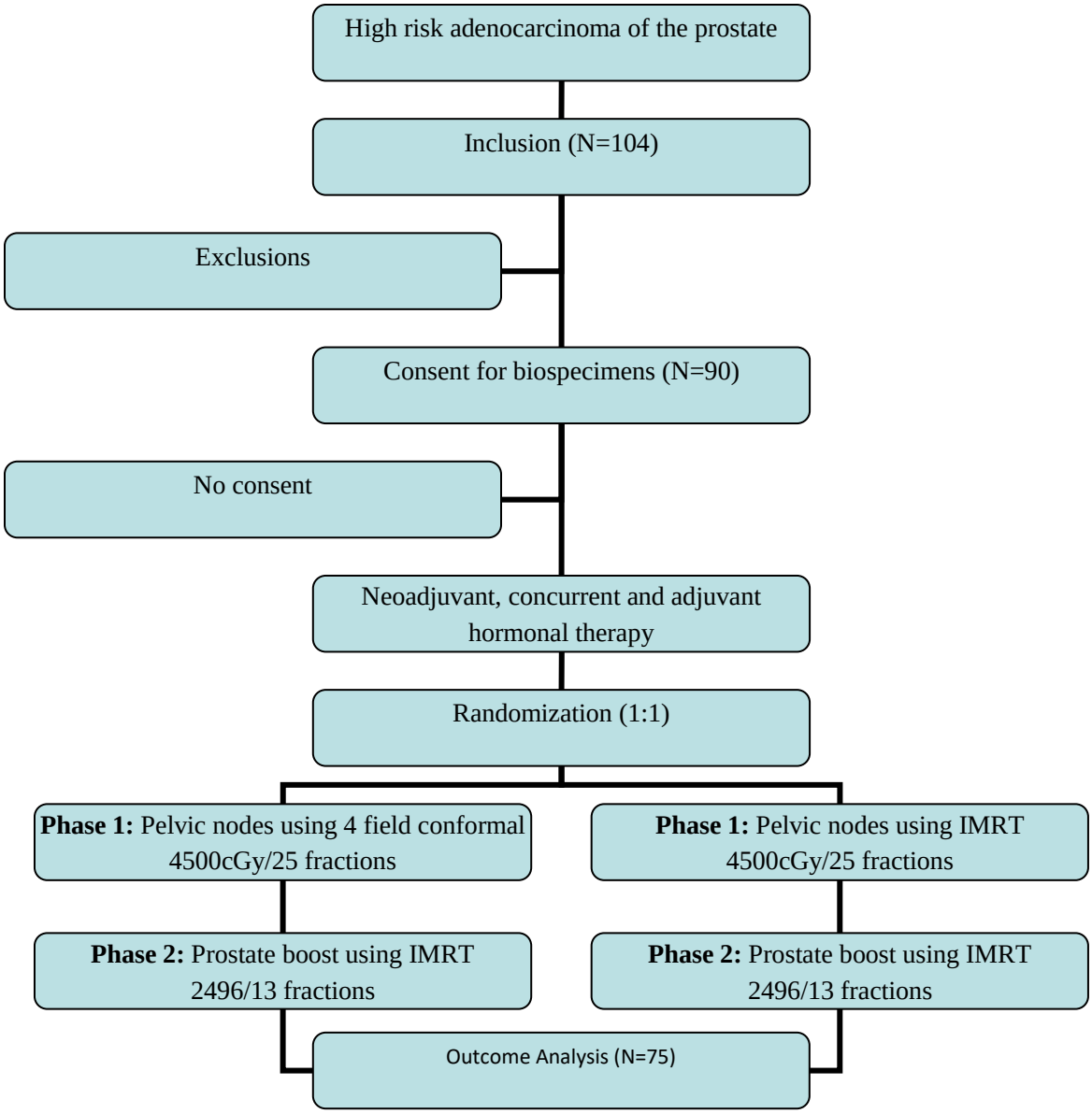


Figure 5. Study Protocol, including patient randomization.

Based on their prostate cancer characteristics shown in Table 2, all patients participated in the clinical trial were randomly assigned (1:1 ratio) to one of the treatment groups (Figure 5): **Group 1:** Received 3D-CRT and neoadjuvant, concurrent, and adjuvant hormones with standard radiation therapy consisting of 4500cGy in 25 fractions to the pelvic nodes using standard 4 field conformal radiation therapy, followed by a

prostate boost of 2496cGy using IMRT; **Group 2:** Received IMRT and neoadjuvant, concurrent, and adjuvant hormones, and pelvic nodal irradiation using IMRT consisting of 4500cGy in 25 fractions to the pelvic nodes, followed by a prostate boost of 2496cGy using IMRT.

Table 2. Baseline prostate cancer characteristics of all patients in the clinical trial.

Characteristic	**IMRT**		**3D-CRT**		**Total**
Number of patients	52		52		104
Clinical Stage	**N**	**%**	**N**	**%**	**N**
Unknown	4	3.8	6	5.8	10
T1	13	12.5	14	13.5	27
T2	27	26.0	19	18.3	46
T3	7	6.7	12	11.5	19
T4	1	1.0	1	1.0	2
PSA value					
≤ 10	15	14.4	15	14.4	30
11-20	15	14.4	14	13.5	29
>20	22	21.2	23	22.1	45
Gleason Score					
6	1	1.0	2	1.9	3
7	18	17.3	19	18.3	37
8-10	33	31.7	31	29.8	64

4.2 Blood collection phase

The blood collection phase was completed, and the samples stored, at the Juravinski Cancer Centre by trained personnel. As shown in Figure 6, each patient was asked to provide three blood samples: the initial sample at the time of radiation therapy simulation (pre-radiation therapy – day 0) and on days 5 (9 Gy) and 25 (45 Gy) of their pelvic radiation therapy. Days 5 and 25 of radiation therapy were selected on the basis of

the descriptions of both early and late waves of circulating cytokine expression during pelvic radiation in previous studies (26,29).

Peripheral blood was drawn from each patient. Standard operating procedures for serum sample collection and EDTA plasma preparation were followed for the blood collection and isolation of EDTA plasma. Blood was collected into BD Vacutainer®, Lavender top tubes containing clot activator and gel from serum separation. Blood samples were processed for plasma isolation (3000xg spin at 4oC) within 30 min at the Juravinski Cancer Centre Clinical Trials Laboratory. All cryotubes were coded and samples were stored at -80 °C until analysis.

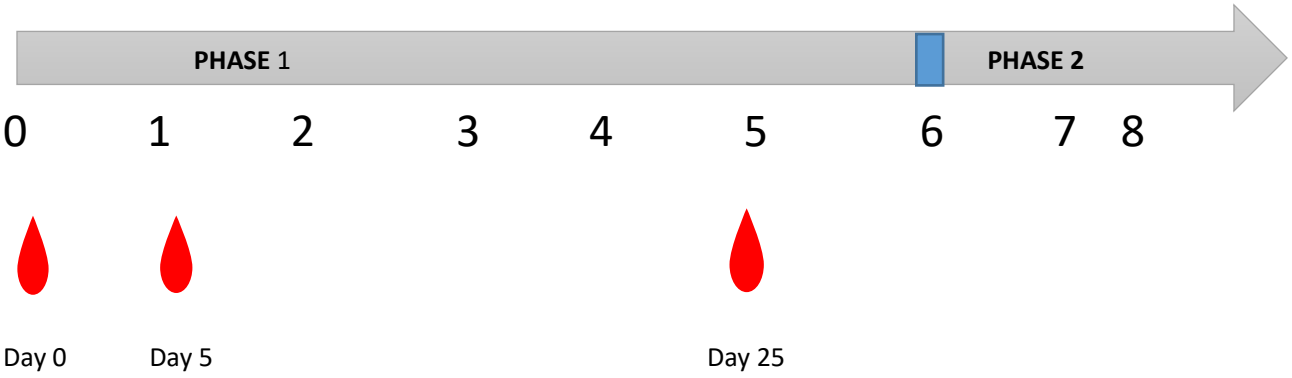


Figure 6. Schematic of sample collection.

The serum samples were placed in a specimen transport box containing dry ice, and transported from the Juravinski Cancer Centre to the Cairns Family Health and Bioscience Research Complex at Brock University for analysis. The serum samples were analyzed for several pro-inflammatory and anti-inflammatory cytokines.

4.3 Cytokine analysis

Inflammatory cytokines, including TNF-α, INF-γ, IL-4, IL-6, IL-8 and IL-10, were analyzed in duplicate, using 6 Milliplex MAGPIX kits (MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay). All assays were performed according to the manufacturer's instructions. The use of Milliplex kits allows for efficient use of time, as several markers can be measured on the same plate. Serum was diluted according to kit-specific instructions (see Appendix x). All samples and kits were brought to room temperature prior to the initiation of the analysis procedure. All analyses were performed in duplicate. Assays were processed over two days. On the first day, all reagents were brought to room temperature and reconstituted. When completed, the plate was sealed with a plate sealer, wrapped in foil, and left to incubate for 16-18 hours on a plate shaker at 4˚C. The following morning, all remaining reagents were removed from the fridge and allowed to warm to room temperature. Cytokine concentrations on each plate were read using xPONENT ® software for the MAGPIX ® analyzer. The intra-assay coefficients of variation for the 6 plates were 10.3, 9.2, 8.7, 6.6, 7.2 and 9.8%, yielding an average intra-assay coefficient of variation of 8.6%. The inter-assay coefficient of variation was calculated by running two quality controls (1 high and 1 low) on each plate and averaging the coefficients from both quality controls from each plate, yielding an average inter-assay coefficient of variation of 7.1%.

4.4 Statistical Analysis

Statistical analyses were performed using SPSS version 23 for Windows and GraphPad Prism 5, SAS Version 9. For all analyses, a p-value ≤ 0.05 was taken as significant. The normality of the dependent variables (inflammatory markers) was

assessed by evaluating three criteria: (i) histogram shape, (ii) Kolmogorov-Smirnov normality tests, and (iii) z-scores for skewness and kurtosis values. Data were not normally distributed for any dependent variable, as histograms were not symmetrical in most cases, normality tests failed, and the z-scores for skewness and kurtosis were outside the accepted range of ± 2.5. As a result, it was decided to use non-parametric tests for the analysis of the cytokine levels.

For the non-parametric analysis, Mann-Whitney tests were used to assess differences in the levels of the inflammatory markers between treatment groups. As no significant differences were found at baseline (day 0) between the groups, the groups were combined for further analysis. To assess changes over time in inflammatory markers for days 0, 5, and 25, a Friedman test for repeated measures was conducted. In case a significant time effect was detected, a Wilcoxon signed-rank test was used for *post hoc* pair-wise comparisons.

Significance was assumed at an alpha level of 0.05 for all tests. Effect sizes (ES) were calculated for the *post hoc* pair-wise comparisons by the following formula: $r = z / \sqrt{n}$, where n = number of observations over the two times points. To examine effect size, the Cohen (1988) criteria were applied – where a value of 0.1 represents a small effect, 0.3 represents a medium effect, and 0.5 represents a large effect (27).

CHAPTER 5: RESULTS

5.1 Differences between 3D-CRT and IMRT (Treatment Effect)

Table 3 shows the concentrations of the various circulating pro-inflammatory cytokines at days 0, 5, and 25. The Mann-Whitney tests revealed that there were no significant differences ($p>0.05$) between the two groups (3D-CRT and IMRT) in the levels of inflammatory markers (IL-4, IL-6, IL-8, IL-10, TNF-α, INF-γ) at any time point (day 0, day 5 and day 25).

Table 3: Mann-Whitney tests for the group differences in the levels of circulating pro-inflammatory markers (cPIC) at each time point (day 0, day 5 and day 25).

cPIC Markers (pg/ml)	**Groups**	**Time Points (days)**					
		Pre-Treatment Day 0		**Day 5**		**Day 25**	
		Mean ± SEM **Median, N**	**M-W U** **Z(p)**	**Mean ± SEM** **Median, N**	**M-W U** **Z(p)**	**Mean ± SEM** **Median, N**	**M-W U** **Z(p)**
IL-4	**3DCRT**	**45.9 ± 8.0** 30.9, 28	431 -0.64 (0.52)	**41.9 ± 7.4** 26.0, 30	509 -0.21 (0.83)	**37.1 ± 7.3** 22.9, 28	506 0.22 (0.82)
	IMRT	**38.7 ± 5.4** 26.9, 34		**39.9 ± 7.8** 25.3, 35		**35.7 ± 4.8** 30.1, 35	
IL-6	**3DCRT**	**2.8 ± 0.5** 2.1, 27	429 0.62 (0.54)	**2.7 ± 0.3** 2.3, 29	393 -0.84 (0.40)	**2.8 ± 0.5** 1.8, 29	507 0.62 (0.53)
	IMRT	**3.2 ± 0.5** 2.5, 29		**3.0 ± 0.6** 1.4, 31		**3.5 ± 0.6** 2.2, 32	
IL-8	**3DCRT**	**7.1 ± 2.6** 4.1, 33	661 0.40 (0.69)	**6.2 ± 1.8** 3.6, 35	657 0.09 (0.93)	**7.8 ± 2.6** 4.2, 34	664 0.20 (0.84)
	IMRT	**5.2 ± 0.7** 3.8, 38		**4.6 ± 0.5** 4.0, 38		**5.6 ± 0.7** 4.4, 38	
IL-10	**3DCRT**	**14.9 ± 2.2** 11.8, 22	259 0.98 (0.33)	**13.4 ± 2.2** 8.7, 23	281 0.93 (0.35)	**12.9 ± 2.4** 9.2, 19	256 0.13 (0.13)
	IMRT	**19.7 ± 3.2** 14.2, 20		**19.9 ± 3.9** 13.4, 21		**18.3 ± 2.6** 14.9, 21	
TNF-α	**3DCRT**	**4.1 ± 0.4** 3.8, 29	456.5 -0.50 (0.61)	**4.0 ± 0.3** 3.7, 29	482.5 -0.15 (0.88)	**3.8 ± 0.3** 2.9, 30	581 1.42 (0.16)
	IMRT	**4.3 ± 0.6** 3.6, 34		**4.8 ± 0.7** 3.3, 34		**4.80 ± 0.6** 4.05, 32	
INF-γ	**3DCRT**	**10.5 ± 1.3** 9.4, 30	609 0.48 (0.63)	**10.6 ± 1.3** 9.2, 33	679 0.40 (0.69)	**10.5 ± 1.5** 7.8, 29	604 0.47 (0.64)
	IMRT	**15.9 ± 2.9** 9.8, 38		**16.1 ± 3.2** 9.8, 39		**15.4 ± 2.8** 9.3, 39	

There was high individual patient variability in the cytokine concentration values as shown by the individual line graphs for each treatment group (see Figures 7-18). For all 6 cytokines, the variation in cytokine concentration was one or more orders of magnitude.

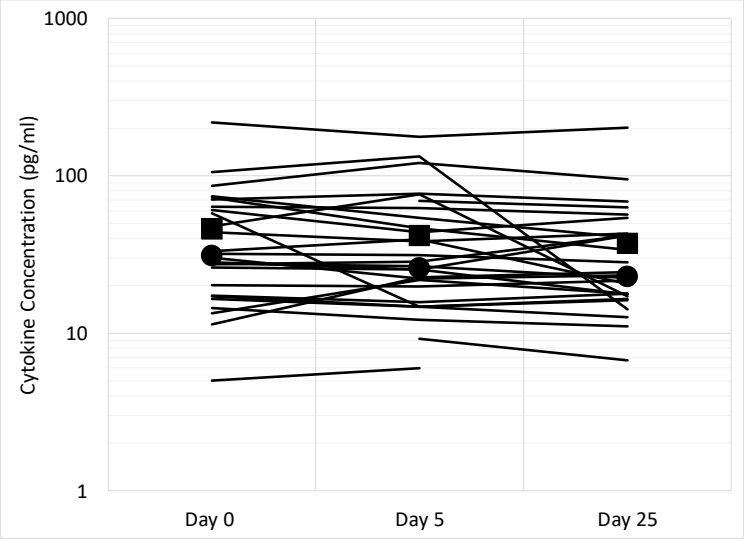


Figure 7. **3D-CRT Group**. IL-4 cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values

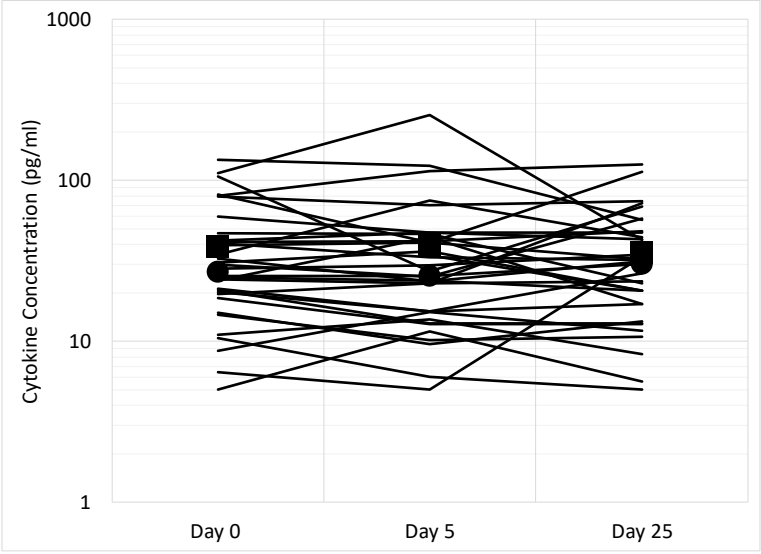


Figure 8. IMRT Group. IL-4 cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

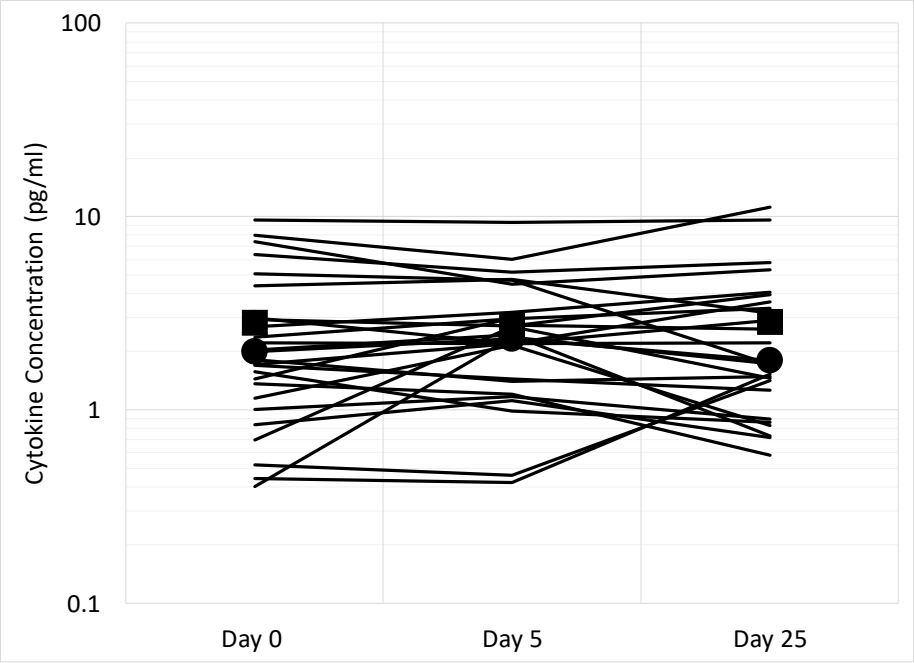


Figure 9. **3D-CRT Group**. IL-6 cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

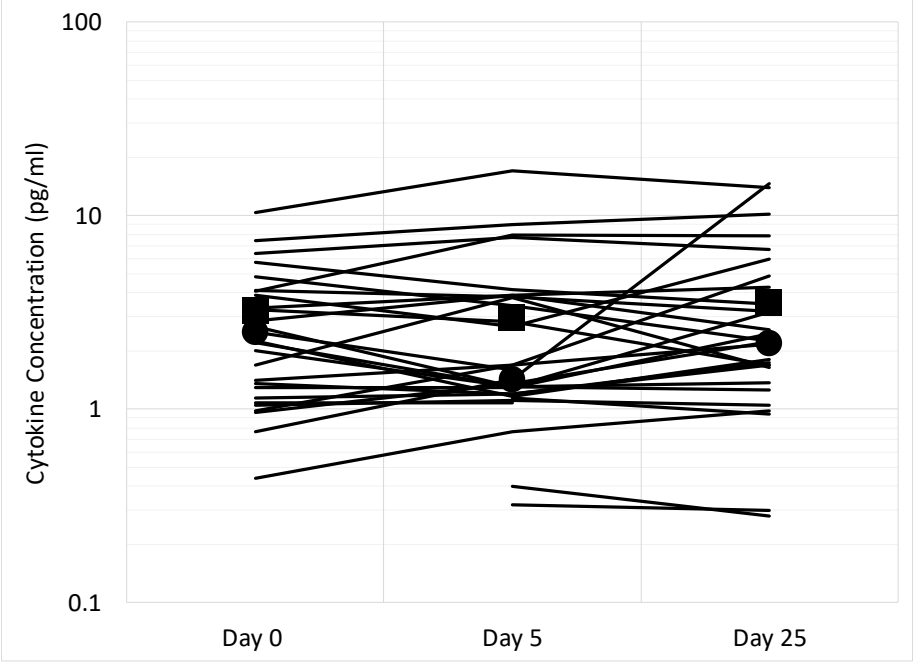


Figure 10. IMRT Group. IL-6 cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

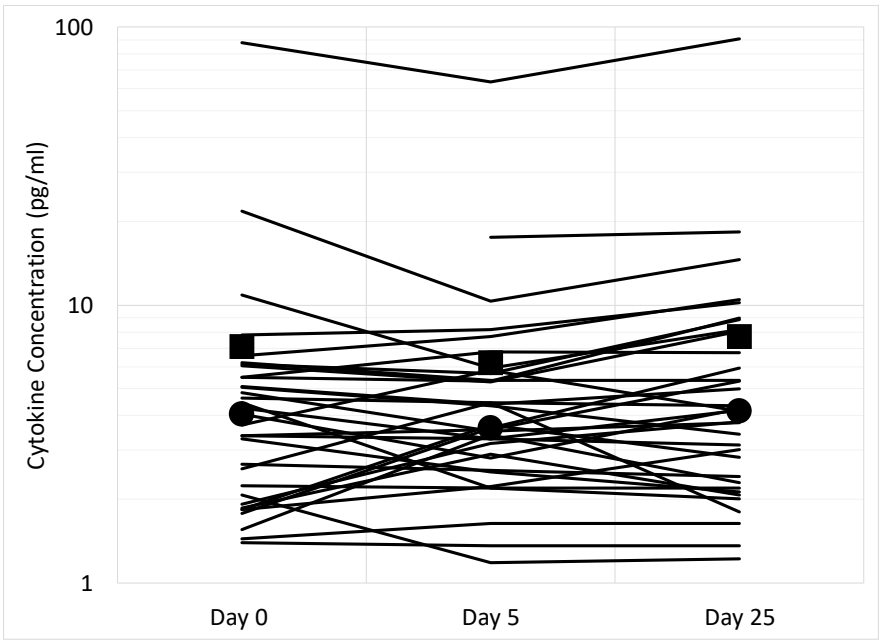


Figure 11. 3D-CRT Group. IL-8 cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

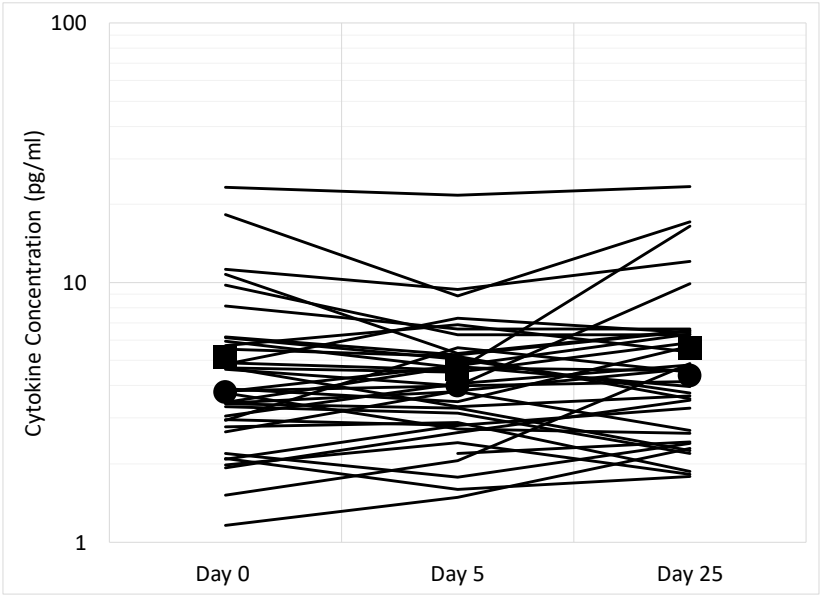


Figure 12. IMRT Group. IL-8 cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

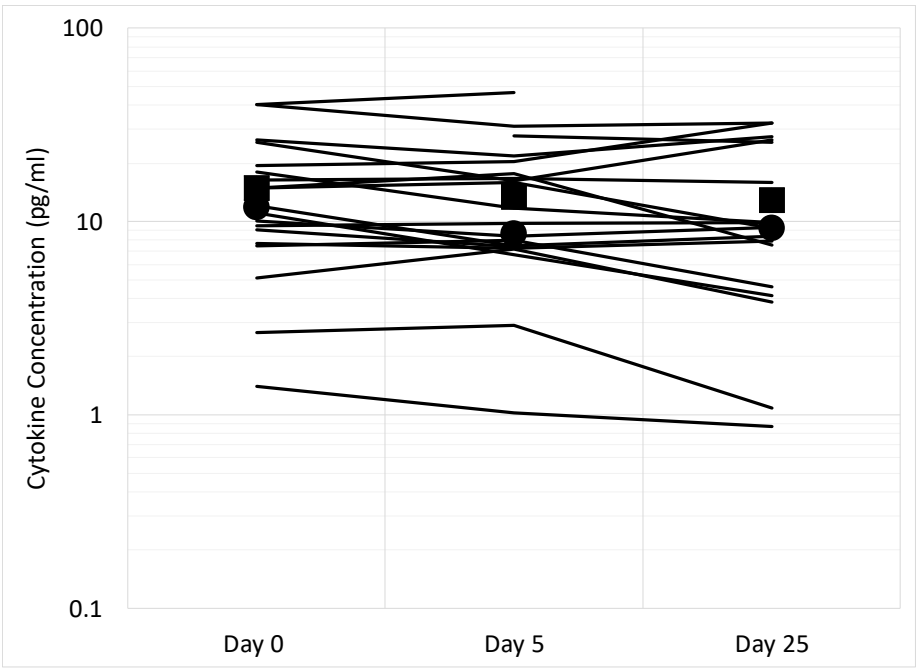


Figure 13. 3D-CRT Group. IL-10 cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

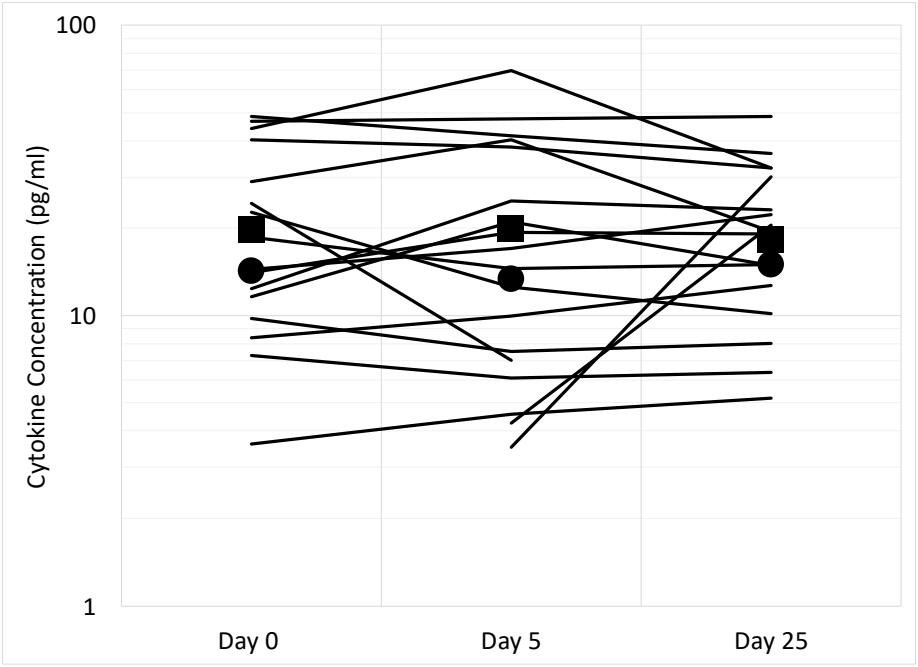


Figure 14. **IMRT Group**. IL-10 cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

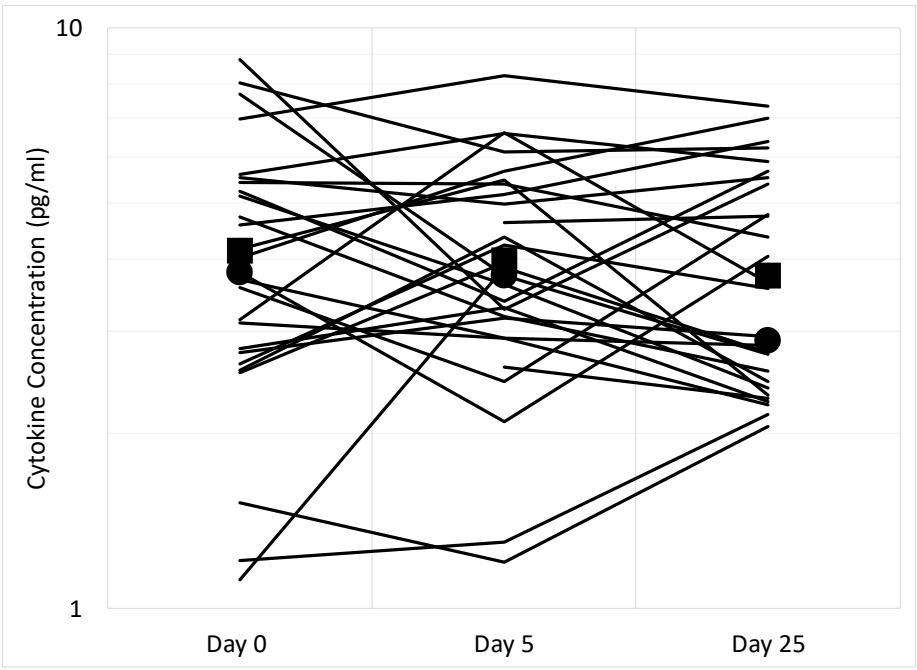


Figure 15. **3D-CRT Group**. TNF-α cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

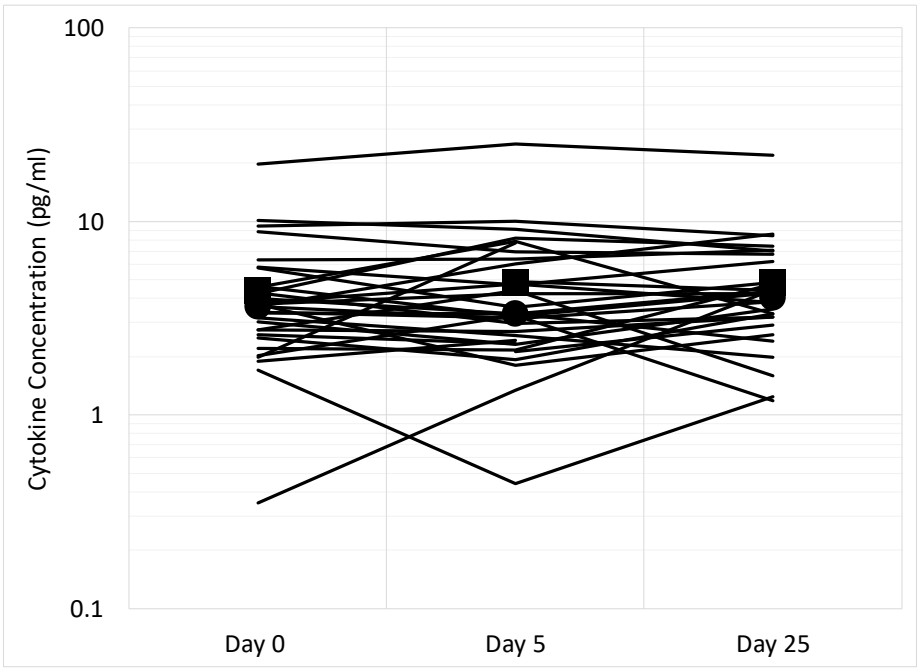


Figure 16. IMRT Group. TNF-α cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

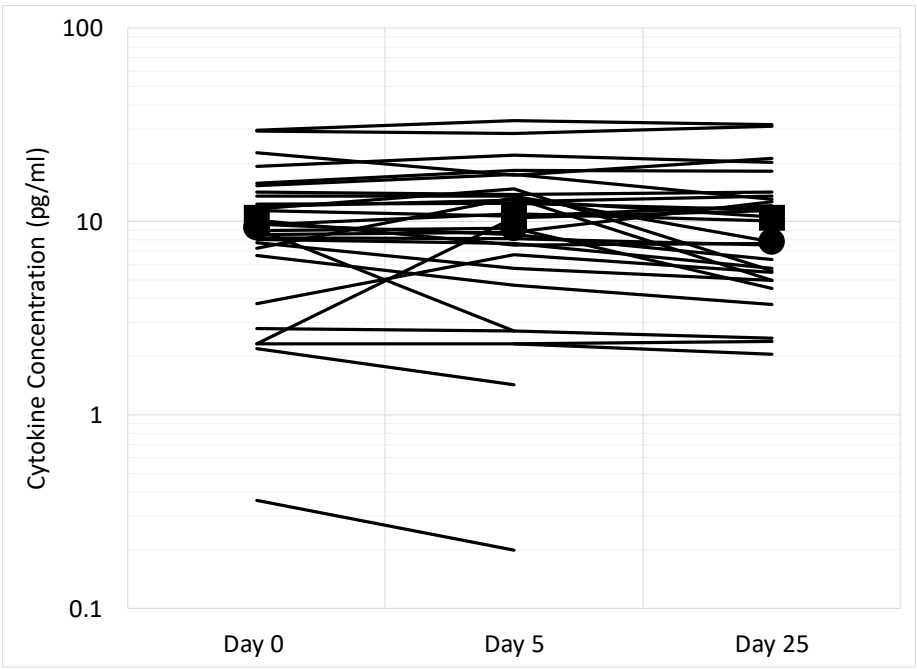


Figure 17. 3D-CRT Group. IFN-γ cytokine concentration (pg/ml) for all participants (n=35) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

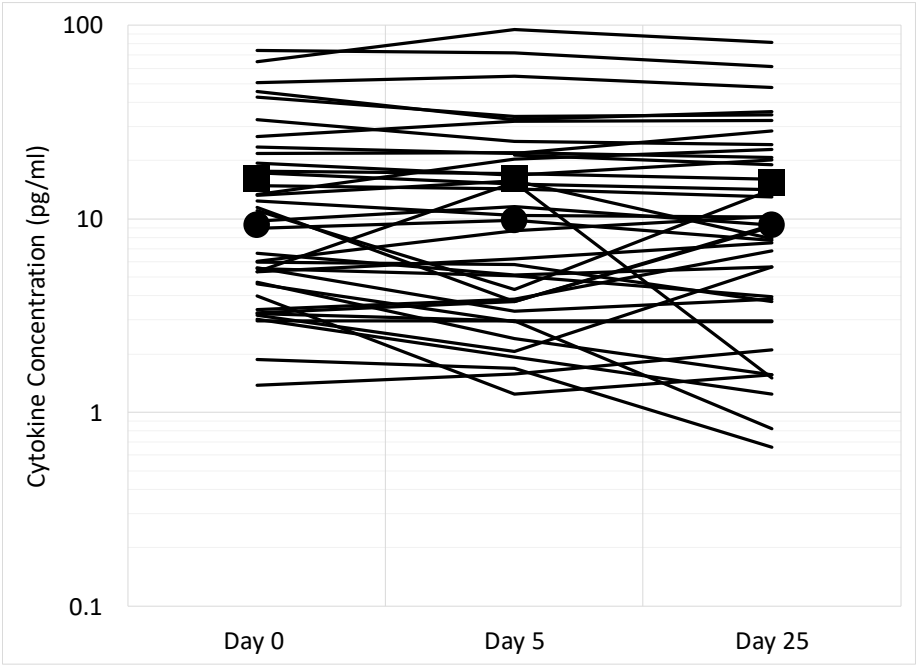


Figure 18. IMRT Group. IFN-γ cytokine concentration (pg/ml) for all participants (n=40) at all time points (Day 0, 5, 25). ▪ = mean values • = median values.

5.2 Time (Dose) Effect

In addition to observing no significance between the two groups, there was also no significant time effect for any of the cytokines when the two groups were examined separately. However, when the treatment groups were combined, a significant time effect was observed for IL-4, which significantly decreased from baseline to day 25 (Figure 19). Further, the effect size of this difference was calculated 0.3. Similar results were found for INF-γ with a significant time effect ($p<0.05$) and a difference from baseline to day 25 of about 2% (Figure 19), and a small effect size of 0.24. According to Table 4 below, there was no significant time effect for the other cytokines.

Table 4. Time effects on the circulating pro-inflammatory markers (cPIC) in the total sample (both groups combined) with post hoc pairwise comparisons.

cPIC Markers	Time Points (days)			χ^2 (DF) p-value, N
	Day 0	Day 5	Day 25	
	Mean ± SEM Median, N	Mean ± SEM Median, N	Mean ± SEM Median, N	
IL-4	**41.9 ± 4.7** 29.2, 62	**40.8 ± 5.4** 25.4, 65	**36.3 ± 4.1** 24.4, 63 [a]	6.80 (2) **0.03*,** 58
IL-6	**3.0 ± 0.3** 2.2, 56	**2.8 ± 0.3** 2.2, 60	**3.2 ± 0.4** 2.1, 61	0.27 (2) 0.87, 50
IL-8	**6.1 ± 1.3** 3.8, 71	**5.4 ± 0.9** 3.8, 73	**6.6 ± 1.3** 4.2, 72	3.79 (2) 0.15, 68
IL-10	**17.2 ± 1.9** 13.2, 42	**16.5 ± 2.2** 10.8, 44	**15.7 ± 1.8** 10.0, 40	1.19 (2) 0.55, 32
TNF-α	**4.3 ± 0.4** 3.7, 63	**4.4 ± 0.4** 3.6, 63	**4.3 ± 0.4** 3.6, 62	0.21 (2) 0.90, 52
INF-γ	**13.6 ± 1.7** 9.3, 68	**13.5 ± 1.8** 9.5, 72	**13.3 ± 1.7** 9.2, 68[b]	7.52 (2) **0.02***, 64

* = statistical significance $p<0.05$ (Friedman test)
a = statistical significance from baseline $p=0.047$ (Wilcoxon Signed Rank Test)
b = statistical significance from baseline $p=0.044$ (Wilcoxon Signed Rank Test)

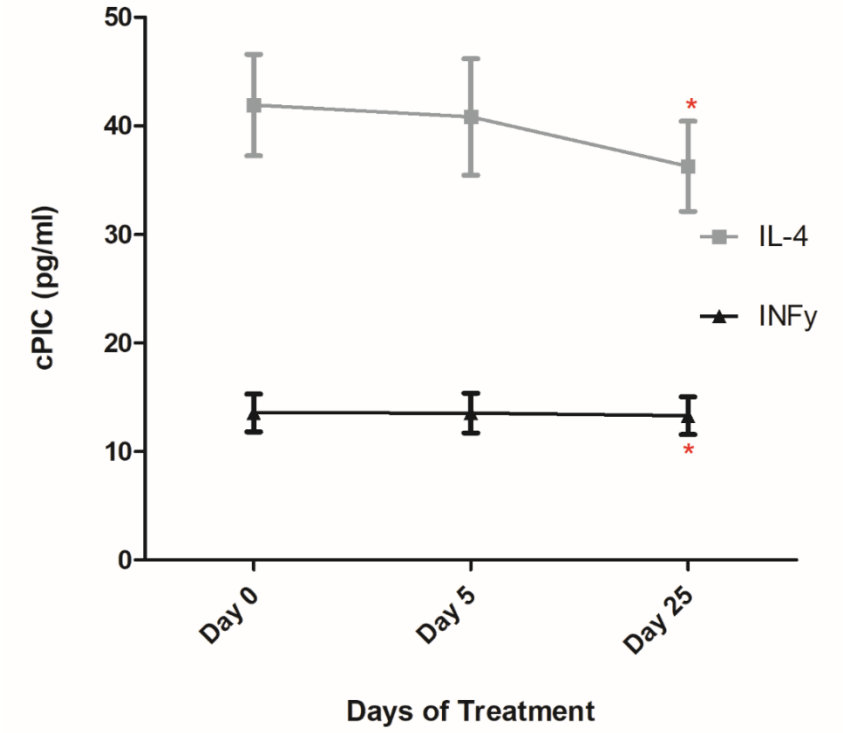


Figure 19. IL-4 and INF-γ levels at day 0 (baseline), 5 and 25. Mean (SEM) values, *statistical significance ($p<0.05$) from baseline (Wilcoxon comparisons).

CHAPTER 6: DISCUSSION

To the best of our knowledge, this is the first study within a randomized trial that examined whether: i) pelvic radiation therapy for high risk prostate cancer influences the levels of circulating cytokines, and ii) whether there are differences in the levels of circulating cytokines between groups of patients treated with 3D-CRT vs. IMRT. The most significant finding was that the circulating pro-inflammatory cytokine concentration levels did not differ between the 3D-CRT and IMRT groups at any of the experimental time periods, i.e., day 0 (pre-treatment), or after either 5 days or 25 days of treatment. In addition, both IL-4 and INF-γ significantly decreased from baseline to day 25 when the results from both radiation therapy groups were combined.

6.1 Baseline Levels of Pro-inflammatory Cytokines

As shown in Table 6, certain cytokines were already elevated at baseline compared to healthy population cohorts. Specifically, IL-4 was much higher in our patients compared to healthy men in previous studies (30,31,39). Likewise, our patients' IL-10 baseline levels were higher than the serum concentrations reported for a healthy Korean cohort of mixed gender (48). For the remaining cytokines, our baseline values were similar to those previously reported in healthy men and prostate cancer patients (Tables 6 and 7). According to previous studies, many cytokines are elevated in the plasma of patients with various cancers, and are produced by various neoplasms in vitro, including prostate cancer. On the other hand, our study shows a considerable variability in the baseline cytokine levels (Figures 7-18), but these levels are not significantly altered by radiation therapy. In addition, it has been suggested that some cytokines may suppress

or change the response of other cytokines (42). This could be taking place in patients receiving radiation therapy and may have influenced the responses we observed in our study. Indeed, a small number of patients, we can see changes in some cytokines such as TNF-α (Figures 15 and 16).

Table 5. Circulating pro-inflammatory markers (cPIC) levels in healthy adults.

	Healthy Cohorts					
	Kim et al. (48)[1]		Chapman et al. (49)[2]	Lopes et al. (30)[3]	Johnke et al. (31)[4]	Abdel-Messeih et al. (39)
cPIC	Mixed Gender (<45 years) Mean±SEM (N=55)	Mixed Gender (>65 years) Mean±SEM (N=55)	Mixed Gender Mean (N=66)	Males range	Males Mean (N=15)	Males Mean±SD (N=20)
IL-4 (pg/ml)	-	-	0.10	0-13.1	-	3.52±0.3
IL-6 (pg/ml)	2.91 ± 0.9	2.57 ± 0.7	0.73	-	~1.2	-
IL-8 (pg/ml)	23.9 ± 4.0	27.6 ± 5.9	7.21	-	-	-
IL-10 (pg/ml)	1.32 ± 0.4	1.58 ± 0.8	0.13	-	-	-
TNF-α (pg/ml)	3.21 ± 0.5	4.94 ± 0.6	5.92	0-20	-	-
IFN-γ (pg/ml)	13.1 ± 3.0	10.3 ± 2.5	13.43	-	-	11.38 ± 1.2

[1]Korean ethnicity; [2] No SEM reported, healthy subjects from all ages and ethnicities;
[3] Only a normal range provided for IL-4 and TNF-α; [4]Approximated from graph, age range 53-79 years.

Table 6. Circulating pro-inflammatory markers (cPIC) levels in prostate cancer patients.

	Studies in Prostate Cancer Patients				
	Present Study	**Christensen et al. (28)[1]**	**Lopes et al. (30)[2]**	**Johnke et al. (31)[3]**	**Abdel-Messeih et al. (39)**
cPIC	Mean±SEM (N=75)	Mean (N=42)	Mean±SEM (N=48)	Mean (N=37)	Mean±SEM (N=20)
IL-4 (pg/ml)	41.9 ± 4.7	-	3.5 ± 1.2	-	7.72 ± 0.65
IL-6 (pg/ml)	3.0 ± 0.3	~1.5-2.0	~3.7-7.7	~1.6-2.7	-
IL-8 (pg/ml)	6.1 ± 1.3	~12.0	-	-	-
IL-10 (pg/ml)	17.2 ± 1.9	~4.0	-	-	-
TNF-α (pg/ml)	4.3 ± 0.4	~10.0	11.7 ± 3.5	-	-
IFN-γ (pg/ml)	13.6 ± 1.7	~0.5-1.0	-	-	8.86 ± 0.61

[1]Approximated from graphs, no tabulated data provided, no SEM reported; [2] Range for IL-6 approximated from graphs; [3] Approximated from graphs;

6.2 Circulating Pro-inflammatory Cytokines in 3D-CRT vs. IMRT Treatments

The findings did not support the hypothesis that cytokine levels would differ between the two radiation treatment protocols, as no statistical differences ($p>0.05$) were found between the groups at any time point (day 0, 5, and 25). The hypothesis stated that cytokine levels would be higher with the 3D-CRT protocol because one of the advantages of IMRT compared to 3D-CRT is the better dosage conformation around the prostate and surroundings organs. However, the IMRT patients had higher concentration levels in most of the circulating pro-inflammatory cytokines markers (except IL-4 and IL-8)

compared to the 3D-CRT group, at all the 3 times points, but the observed differences were not statistically significant.

To our knowledge, there are no other prostate cancer studies that have compared cytokine concentrations between 3D-CRT and IMRT patients. Previous studies have compared 3D-CRT and IMRT patients in terms of toxicity and dosimetry, but did not measure cytokine levels (12,21,37).

6.3 Time (Dose) Effect of Radiation Therapy on Circulating Cytokines

Since there were no statistical differences in the cytokine concentrations between the groups across all time points, the groups were combined for the repeated measures analysis. A significant time effect was found for IL-4 and INF-γ, with *post hoc* pair-wise analysis showing a significant decrease at day 25 compared to baseline ($p<0.05$). Previously, Lopes et al. found only an increase in IL-6 during radiation therapy in patients with prostate cancer, with no change in IL-2, IL-4, IL-5, TNF-α, leukemia inhibitory factor, and macrophage inflammatory protein 1-α (30). Likewise, in the study of Chistensen et al., no significant difference ($p>0.05$) was observed in cytokines, including granulocyte macrophage colony stimulating factor, IL-10, IL-1α, IL-2, IL-6, IL-8, and TNF-α, before and during radiation therapy in a definitive (intact prostate) group receiving IMRT. However, when combined with a post-operative IMRT group, there was a significant increase in IL-6 and IFN-γ ($p<0.05$), which is in contrast to our study where IFN-γ significantly decreased 25 days into treatment (28).

6.3.1 IL-4

IL-4 has an important role in regulating inflammatory and cell-mediated immune responses. IL-4 acts as an immune-suppressor of cancer immunity and promotes cancer develop-ment. In the past, only a few studies have investigated serum IL-4 levels in prostate cancer patients. It has been suggested that elevated IL-4 may be linked to disease evolution to castrate resistance (38). Abdel-Messeih et al. recently evaluated 20 patients with prostate cancer, who were subjected to radiation therapy post-prostatectomy, with a total dose of 66 Gy in 33 fractions (5 sessions/week) for 7 weeks. The level of inflammatory cytokines IL-4, IL-5 and INF-γ in post-radiation therapy patients were significantly elevated compared to both controls and prostate cancer patients (39). Our study, on the other hand, found a significant time effect for IL-4 during radiation therapy, which decreased 25 days into treatment. Cytokine production is time-dependent, peaking usually at 4–24 hours after irradiation, followed by a subsequent decrease to baseline levels within 24 hours to a few days for some type of cancers (38).

6.3.2 TNF-α

Since TNF-α has been shown to be expressed by prostate cancer (40), and plays a major role in the inflammatory process, we would have expected TNF-α to increase significantly during radiation therapy. However, neither our study, nor the study of Lopes et al. (30), found an increase in TNF-α during radiation therapy. In addition, Lopes et al., in their examination of IL-6, IL-2, IL-4, IL-5, TNF-α, macrophage inflammatory protein 1-α, and leukemia inhibitory factor levels of prostate cancer patients treated with 3D-CRT, found a significant increase of IL-6. They speculated that because IL-6 has also been described as an anti-inflammatory cytokine that suppresses the activity of pro-

inflammatory mediators, the rise of IL-6 levels following radiation therapy could have been responsible for the suppression of other cytokines associated with the acute phase reaction (30).

6.3.3 IL-6

Jonke et al., in their examination of the circulating levels of IL1-β, IL-6 and TGF-β in prostate cancer, compared the cytokines levels between a group treated with radiation therapy alone, a group treated with radiation therapy plus androgen therapy, and a healthy control group (31). They found that IL6 and IL-1β increased immediately during radiation therapy for both the radiation therapy groups, with and without androgen deprivation therapy. These cytokines peaked after 1 to 2 weeks of radiation therapy before returning to pre-therapy levels. They also observed an immediate decrease in TGF-β during radiation therapy, with two distinct waves of elevation, one at 1-2 weeks, and a second, 5-6 weeks into the radiation therapy (31). Lopes et al. found a significant rise for IL-6 at day 15 of radiation therapy ($p<0.0049$) and a decline at day 30, to levels similar to pre-treatment levels (30). Kovacs et al. described the elevated levels of cytokines in prostate cancer patients receiving radiation therapy in the form of cyclic waves, that were associated with accumulating doses of radiation therapy (26). However, none of these effects were observed in our cohort of patients receiving either IMRT or 3D-CRT treatment.

In our study, we selected two-time points during radiation therapy based on descriptions of both early and late waves of circulating cytokine expression during pelvic radiation in other studies (28,31). The study by Christensen et al. obtained blood samples every week during radiation therapy, but their study didn't find any significant changes in

cytokine expression during radiation therapy for an “intact” prostate group treated with IMRT (28). Thus, the cyclic wave response of cytokine expression during radiation therapy appears to indicate that more frequent measurements of cytokine concentrations need to occur if we are to understand this time dependant response.

One limitation of our study is that having only two-time measurements (days 5 and 25 of treatment) may have not produced the resolution in time needed to capture these responses. The incentive for this choice of sampling times was to determine whether, i) early cytokine levels could predict radiation therapy toxicity and ii) whether cytokine levels at the time of completion of radiation therapy correlate with toxicity. Likewise, DiMaggio and colleagues also found that men with prostate cancer undergoing long-term androgen deprivation therapy did not demonstrate elevated levels of inflammatory cytokines compared to age and disease-matched controls (41).

Johnke et al. (31) compared two groups of prostate cancer patients undergoing radiation therapy: one group with androgen deprivation therapy, and a second group without androgen deprivation therapy. They examined different cytokine levels and found that following initiation of radiation therapy, both patient groups demonstrated an immediate elevation of the pro-inflammatory cytokines IL-1β and IL-6 in their plasma. They described that the magnitude of cytokine expression was noticeably different in the group that received androgen deprivation therapy. More specifically, mean plasma levels of IL-1β and IL-6 significantly were significantly elevated following two months of ADH when compared to pre ADH values (31). However, Lopes et al. didn’t find any significance differences in cytokine levels, except for the IL-6. They explained that one possibility might be the concurrent hormone therapy (30), suggesting that the magnitude

of the cytokine levels in patients with prostate cancer treated with radiation therapy appeared to be affected by the addition of the hormone therapy. Our study was not designed to investigate the impact of androgen deprivation therapy, but rather the effects of the radiation therapy in patients that receive this treatment. However, in future studies, it would be useful to collect blood samples from the patients before initiating the androgen deprivation therapy.

6.4 Strengths and Limitations

To our knowledge, this is the first study to examine the response of cytokine expression from prostate cancer patients treated with either 3D-CRT vs. IMRT. The study has a number of strengths and limitations that are important to discuss, and are noteworthy for planning future studies.

In terms of strengths, randomized trials are considered the gold standard for clinical study design in that such a design reduces selection bias. Patients participating in this trial were randomly allocated to either 3D-CRT or IMRT. A second strength was the relatively large sample size. Previous studies describing the role of circulating pro-inflammatory cytokines during radiation therapy on prostate cancer have used smaller sample sizes.

The large individual variability in the cytokine concentrations at baseline could be considered a limitation. Furthermore, we found that the levels of two cytokines (IL-4 and IL-10) were already significantly elevated compared to levels found in normal, healthy men of this age group. It is possible that these elevated levels may have altered the immune response of the patients to the radiation therapy. According to previous studies,

many cytokines are elevated in the plasma of patients with various cancers, including prostate cancer, and that the levels are relatively invariant during radiation therapy (26, 40). In addition, it has been suggested that some cytokines may suppress or change the response of other cytokines (30), which may have influenced the responses we observed in our study.

Potential confounders in our study include lifestyle choices, medications and comorbidities. These conditions, which have been previously found to contribute to systemic inflammation (e.g., severe osteoarthritis or connective tissue disorders), can impact baseline cytokine variability (33). For example, Druzgal et al. reported increases in IL-6 and hepatocyte growth factor (HGF) in cancer patients who relapsed, but also in patients who had developed inflammatory conditions. In addition, longitudinal increases in serum IL-6, IL-8, vascular endothelial growth factor, and human growth factor have been significantly associated with a decreased cause-specific survival in patients with locally advanced oropharyngeal squamous cell carcinoma (27). For instance, a number of patients smoked during and after treatment. The impact of this habit or other co-morbidities, on inflammatory cytokines like IL-6 or IL-8, was not accounted for (43).

In terms of design limitations, an ideal design would include a healthy, age and weight-matched control group to make comparisons between the healthy population and cancer patients. Secondly, since cytokine levels behave in the form of cyclic waves (26,31), additional time points would allow better characterization of the cytokine response. However, since every study seemed to measure the cytokine concentrations at different time points, it was difficult to ascertain the appropriate time points during radiation therapy to take the blood samples. However, since every study seemed to

measure the cytokine concentrations at different time points, it was difficult to ascertain the appropriate time points during radiation therapy to take the blood samples. Lastly, it would have been ideal to collected blood samples before androgen therapy in order to understand how hormonal therapy affects the cytokine expression.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

In recent years, studies have focused on elucidating clinically useful biomarkers of radiation therapy-induced toxicity, with the goal of identifying a patient's radio-sensitivity profile, which could lead to the development of personalized treatments, and ultimately, to improved local control of radiation to minimize tissue damage in surrounding tissues, and improved quality of life.

The concept of investigating the levels of circulating pro-inflammatory cytokines as potential biomarkers of inflammation and radiation toxicity is well supported by a significant number of clinical studies. Unfortunately, it is difficult to compare, and to draw conclusions from these previous due to differences in methodological design, subject pools, investigative techniques, and findings. As a result, it has not been possible to get a complete picture of the response of circulating pro-inflammatory cytokines to radiation therapy, and therefore, their potential as useful biomarkers of toxicity.

The present study was undertaken as part of a larger clinical trial aimed at examining the efficacy of IMRT as the protocol of choice in the treatment of prostate cancer. The work described here was designed to examine the response of various cytokines to radiation therapy, and to examine the levels of circulating cytokines in relation to radiation protocol (IMRT vs. 3D-CRT), radiation dose to the prostate, nodal areas, and normal tissues, as well as measures of patient quality of life. To this end, we utilized a randomized, control trial, that involved 75 patients, divided into two groups,

and assessed cytokine levels at 3 time points, namely, before and during the radiation therapy.

Our study suggests that, in high risk prostate cancer patients, who are treated with androgen deprivation therapy, there is no statistically significant difference in the mean levels of circulating pro-inflammatory cytokines between patients receiving radiation treatment via 3D-CRT vs IMRT. However, an analysis of the pooled data from all patients revealed statistically significant changes over time, which indicated that IL-4, and perhaps IFN-γ, decreased with cumulative radiation therapy dose, independent of radiation therapy technique.

The present study was not able to observe any significant evidence that radiation therapy technique modulates cytokine levels in this group of patients. Often, a low statistical power, due to a small sample size, is the reason for an inability to detect significant differences in results; however, the present study is one of the largest to date to measure cytokine expression on prostate cancer patients treated with 3D-CRT vs. IMRT, and thus, should have provided adequate statistical power.

Another issue that influences the potential to find statistical differences involves an increased variability in the data due to biological variation between subjects, differences in their response to a perturbation of the system, and our inability to detect change. The issues to examine here include i) higher cytokine levels at baseline (time 0), ii) differences in the cytokine response to variations in radiation dose, iii) sub-optimal times of sampling, both in terms of diurnal variation, and in the choice of time points during treatment, and iv) a mismatch between the sensitivity of the biochemical assay to

detect changes in the levels of the cytokines compared to the actual changes that occurred.

At present, the cytokine response to radiation therapy is poorly understood. As indicated above, the gathering and analyzing of data concerning the cytokine expression in patients receiving radiation therapy is complicated by several factors. Future studies should control for the following: i) the effects of baseline characteristics of the patient population, such as age, differences in the tumor burden and medical comorbidities, ii) diurnal variations in cytokines levels, iii) ongoing patho-physiological stressors, including infection, cancer, and trauma, and iv) the use of prescribed and non-prescription medications, especially anti-inflammatory drugs. Further, we need to determine the impact of other confounders, including age, radiation-induced changes in patient weight, the use of androgen hormone therapy as part of the treatment regimen, and overall individual patient radio-sensitivity.

It is anticipated that the results of the present study will provide evidence whether cytokine levels at baseline, or during radiation therapy are associated with radiation therapy toxicity, the efficacy of the IMRT protocol (compared to the 3D-CRT protocol), and the relationship of cytokines as a biomarker that might be used to develop individual radiation therapy protocols to enhance the patients' quality of life.

In conclusion, this study has provided novel information concerning cytokine responses to radiation therapy. Future trials will need to address the concerns raised above, including sample size, control groups, well defined criteria for sampling periods and protocols based on the normal diurnal variation of cytokines, and improved biochemical assays with an increased sensitivity to the changes in cytokine level, will

allow future researchers to ascertain the efficacy of assessing cytokine responses as a tool for improving the potential role of cytokines as biomarkers of radiation therapy toxicity in patients treated for prostate cancer.

REFERENCES

1. Prostate Cancer Stats. Canadian Cancer Society (www.cancer.ca)

2. Perez C. and Brady L. Principles and Practices of Radiation Oncology, 5th Edition. Chapter 62, 63; Lippincott William and Wilkins. 2008. pp.1439-1518;

3. Leibel P. Textbook of Radiation Oncology, 2nd Edition. Part 5: Cancer of the Prostate Cancer; Mack Roach and Kent Wallner;2004. pp. 959-1029

4. Nicol, A.M., Warde, P. and Bristow, R.G. "Optimal treatment of intermediate-risk prostate carcinoma with radiotherapy" Cancer 104: 891-905, 2005.

5. Christensen E., Evans K.R., Ménard C., Pintilie M. and Bristow R. "Practical approaches to proteomic biomarkers within prostate cancer radiotherapy trials". Cancer Metastis Rev 27: 375–385, 2008.

6. Menard, C., Johann, D., Lowenthal, M., Muanza, T., Sproull, M., Ross, S., Gulley, J., Petricoin, E., Coleman, C. N., Whitely, G., Liotta, L., and Camphaussen, K. "Discovering clinical biomarkers of ionizing radiation exposure with serum proteomic analysis". Can Res 66: 1844-1850, 2006.

7. Seruga, B., Zhang, H., Bernstein, L.J. and Tannock, I.F. "Cytokines and their relationship to the symptoms and outcome of cancer". Nat Rev Cancer, Vol. 8 Issue 11, p887-899. 13p 2008.

8. Ellsworth, S.G., Rabati,c B.M., Chen, J., Zhao, J., Campbell, J., Wang, W., Pi, W., Stanton, P., Matuszak, M., Jolly, S., Miller, A. and Kong, F.M. "Principal component analysis identifies patterns of cytokine expression in non-small cell lung cancer patients undergoing definitive radiation therapy". PLOS One. 12 (9): e0183239, 2017.

9. Hall, E.J. “Clinical Response of Normal Tissue”. In: Radiobiology for the Radiologist, 5th edition, New York: Lippincott Williams & Wilkins, 2000. pp. 339-360

10. Zelefsky, M.J., Xin Pei, X., Chou, J.F., Schechter, M., Kollmeier, M., Cox, B., Yamada, Y.Fidaleo, A., Sperling, D., Happersett, L. and Zhang, Z. “Dose Escalation for Prostate Cancer Radiotherapy: Predictors of Long-Term Biochemical Tumor Control and Distant Metastases–Free Survival Outcomes”. Eur Urol 60:1133-1139, 2011.

11. Al-Mamgani, A., Heemsbergen, W., Peeters, S. and Lebesque, J. “Role of Intensity-Modulated Radiotherapy in reducing toxicity in dose escalation for localized Prostate Cancer”. International Journal Radiation Oncology Biol Phys 73: 685-691, 2009.

12. Fenoglietto, P., Laliberte, B., Allaw, A., Ailleres, N., Idri, K., Hay, H.M., Moscardo, L.C., Gourgou, S., Dubois, J-B. and Azria, D. “Persistently better treatment planning results of intensity-modulated (IMRT) over conformal radiotherapy (3D-CRT) in prostate cancer patients with significant variation of clinical target volume and/or organ-at-risk”. Radiother Oncol 88: 77-87, 2008.

13. Yu, T., Zhang, Q., Zheng, T., Shi, H., Liu, Y., Feng, S., Hao, M., Ye, L., Wu, X. and Yang, C. “The Effectiveness of Intensity Modulated Radiation Therapy versus Three-Dimensional Radiation Therapy in Prostate Cancer: A Meta-Analysis of the Literatures”. PLOS ONE 2016, doi: 10.1371/journal.pone.0154499.

14. Pearlstein, K.A. and Chen, R.C. “Comparing Dosimetric, Morbidity, Quality of Life, and Cancer Control Outcomes After 3D Conformal, Intensity-Modulated, and Proton Radiation Therapy for Prostate Cancer”. Semin Radiat Oncol 23: 182-190; 2013.

15. Burnet, N., Thomas, S., Burton, K. and Jeffries, S. “Defining the tumor and target the volumes for radiotherapy”. Cancer Imag 4: 153-161, 2004.

16. Sharifi, N., Gulley, J. and Dahut, W. “Androgen Deprivation Therapy for Prostate Cancer”. Jama. 2005;294(2):238-244. doi:10.1001/jama.294.2.238

17. Bolla, M., De Reijke, T.M., Zurlo, A. and Collette, L. “Adjuvant hormones in locally advanced and localized prostate cancer: three EORTC trials”. Front Radiat Ther Oncol. (2002); 36:81-6

18. Zelefsky, M.J., Chan, H., Hunt, M., Yamada, Y., Shippy, A. and Amols, H. “Long-term outcome of high dose intensity modulated radiation therapy for patients with clinically localized prostate cancer”. J Urol 176: 1415-1419, 2006.

19. Zelefsky,M.J, Fuksa, Z., Happersettb, L., Leea, H.J., Lingb, C.C., Burmanb, Hunt, B.C.M., Wolfea, T., Venkatramanc, E.S., Jacksonb, A., Skwarchukb, M. and Leibela, S.A. “Clinical experience with intensity modulated radiation therapy (IMRT) in prostate cancer”. Radiother Oncol 55: 241-249, 2000.

20. Mok,H, Crane, C.C., Palmer, M.B., Briere, T.M., Beddar, S., Delclos, M.E., Krishnan, S. and Das P.; “Intensity modulated radiation therapy (IMRT): differences in target volumes and improvement in clinically relevant doses to small bowel in rectal carcinoma”. RadiatOncol. 6: 63, 2011, doi: 10.1186/1748-717X-6-63.

21. Viani, G.A., Viana,B.S., Martin, J.E.C., Rossi, B.T., Zuliani,G. and Stefano, E.J. “Intensity-modulated radiotherapy reduces toxicity with similar biochemical control compared with 3-dimensional conformal radiotherapy for prostate cancer: A randomized clinical trial”. Cancer 122: 2004-2011, 2016.

22. Zhang, J-M. and An, J. “Cytokines, Inflammation and Pain”. Int Anesthesiol Clin 45: 27-37, 2007.

23. Gridley, D.S., Slater, J.D. and Yonemoto, L.T. “Pilot study of cytokine profiles in prostate cancer patients undergoing proton or conventional radiotherapy”. Int J Oncol 1996; 8:175–81

24. Dranoff, G. “Cytokines in cancer pathogenesis and cancer therapy”. Nat Rev 4: Vol. 4 Issue 1, p11-22. 12p; 2004.

25. Rubin, P., Johnston, C.J., McDonald, S. and Finkelstein, J.N. “A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis”. Int J Radita Oncol Biol Phys 33:99-109, 1995.

26. Kovacs, C.J., Daly, B.M., Evans, M.J., Johnke, R.M., Lee, T.K., Karlsson, U.L., Allison, R., Eaves, G.S. and Biggs, L.M. “Cytokine profiles in patients receiving wide-field+prostate boost radiotherapy (xRT) for adenocarcinoma of the prostate”. Cytokine 23: 151–163, 2003.

27. Druzgal, C.H., Chen, Z., Yeh, N.T., Thomas, G.R., Ondrey, F.G., Duffey, D C., Vilela, R.J., Ende, K., McCullagh, L., Rudy, S.F., Muir, C., Herscher, L.L., Morris, J.C., Albert, P.S. and Waes, C.V. “A pilot study of longitudinal serum cytokine and angiogenesis factor levels as markers of therapeutic response and survival in patients with head and neck squamous cell carcinoma”. Head & Neck, 27: 771-784, 2005.

28. Christensen, e., Pintilie, M., Evans, K.R., Lenarduzzi, M., Ménard, C., Catton, C.N., Diamandis, E.P., and Bristow, R.G. “Longitudinal Cytokine Expression during IMRT for Prostate Cancer and Acute Treatment Toxicity”. Clin Can Res 15:5576–83, 2009.

29. Dirksen, S. R., Kirschner, K.F. and Belyea, M.J. “Association of Symptoms and Cytokines in Prostate Cancer Patients Receiving Radiation Treatment”. Biol Res Nurs 16: 250-257, 2014.

30. Lopes, C.O.and Callera, F. “Three- Dimensional Conformal Radiotherapy in prostate cancer patients: Rise in interleukin 6 (IL-6), but not IL-2, IL-4, IL-5, Tumor Necrosis Factor-a, Macrophage Inflammatory Protein 1-alpha and LIF levels”. Int. J. Radiat Oncol Biol. Phys;82: 1385–1388, 2012

31. Johnke, R.M., Edwards, J.M., Evans, M.J., Nangami, G.N., Bakken, N.T.G., Kilburn, J.M., Lee, T-K., Allison, R.R., Karlsson, U.L. and Arastu, Hyder H.Arastu, “Circulating Cytokine Levels in Prostate Cancer Patients Undergoing Radiation Therapy: Influence of Neoadjuvant Total Androgen Suppression”. In vivo 23: 827-834, 2009

32. Maggio, Marcello; Blackford, Amanda; Taub, Dennis; Carducci, Michael; Ble, Alessandro; Metter, E Jeffrey; Braga-Basaria, Milena; Dobs, Adrian; Basaria, Shehzad “Circulating inflammatory cytokine expression in men with prostate cancer undergoing androgen deprivation therapy”. Journal of Andrology; Vol. 27 Issue 6, p725-728, 2006.

33. Tanji, N., Kikugawa, T., Ochi, T., Tagushi, S., Sato, H., Sato, T., Sugahara, T., Hamada, H., Asai, S. and Matsumoto, A. “Circulating Cytokine Levels in Patients with Prostate Cancer: Effects of Neoadjuvant Hormonal Therapy and External-beam Radiotherapy”. Antican Res 35: 3379-3383, 2015.

34. Ghasemi, A and Zahediasl, S. “Normality Tests for Statistical Analysis: A Guide for Non-Statisticians”. Int J Endocrinol Metab. 2012;10(2):486-489. DOI: 10.5812/ijem.3505

35. Cohen, J.W. (1988). Statistical power analysis for the behavioral sciences (2nd Edition); p.79-81; Hillsdale, NJ: Lawrence Erlbaum Associates

36. Pallant, J. "SPSS Survival Manual – "A step by step guide to data analysis using SPSS" 4th Edition. Part Five Statistical techniques to compare groups. Allen & Unwin, 2011. pp. 203-297

37. Michalski, J.M., Yan, Y., Watkins-Bruner, D., Bosch, W., Winter, K., Galvin, J.M., Bahary, J-P., Morton, G.C., Parliament, M.B. and Sandler, H.M. "Preliminary Toxicity Analysis of 3DCRT versus IMRT on the High Dose Arm of the RTOG 0126 Prostate Cancer Trial". Int J Radiat Oncol Biol Phys. 87: 10.1016/j.ijrobp.2013.07.041, 2013

38. Goldstein, R., Hanley, C., Morris, J., Cahill, J.D., Chandra, A., Harper, P., Chowdhury, S., Maher, J. and Burbridge, S. "Clinical Investigation of the Role of Interleukin-4 and Interleukin-13 in the Evolution of Prostate Cancer". Cancers 3: 4281-4293; doi:10.3390/cancers3044281, 2011.

39. Abdel-Messeih, P.L., Nosseir, N.M. and Bakhe, S.H. "Evaluation of inflammatory cytokines and oxidative stress markers in prostate cancer patients undergoing curative radiotherapy". Cent Eur J Immunol 42: 68–72, 2017.

40. Adler, H.L., McCurdy, M.A., Kattan, M.W., Timme, T.L., Scardino, P.T. and Thompson, T.C. "Elevated levels of circulating interleukin-6 and Transforming growth factor-β1 in patients with metastatic prostatic carcinoma" J Urol 161: 182-187, 1999.

41. Di Maggio, F.M., Minafra, L., Forte, G.I., Cammarata, F.P., Lio, D., Messa, C., Gilardi, M.C. and Bravatà, V. "Portrait of inflammatory response to ionizing radiation treatment". J Inflamm Vol. 12, 14 2015.

42. Michalaki, V., Syrigos, K., Charles P. and Waxman, J. “Serum levels of IL-6 and TNF-a correlate with clinicopathological features and patient survival in patients with prostate cancer”. Brit J Can 90: 2312–2316, 2004.

43. Allen, C., Duffy, A.C., S., Teknos, T., Islam, M., Chen, Z., Albert, P.S., Wolf, G., and Waes, C.V. “Nuclear factor-kB-related serum factors as longitudinal biomarkers of response and survival in advanced oropharyngeal carcinoma”. Clin Can Res 13: 31823190, 2007.

44. Torrealba, N., odriguez-Berriguete, G., Fraile, G.B., Olmedilla, G., Martinez-Onsurbe, P., Guil-Cid, M., Paniagua R. and Royuela, M. “Expression of several cytokines in prostate cancer: Correlation with clinical variables of patients. Relationship with biochemical progression of the malignance”. Cytokine 89: 105-115, 2017.

45. Gaudreau, P.O., Stagg, J., Soulieres, D. and Saad, F. “The Present and Future of Biomarkers”, In: Prostate Cancer: Proteomics, Genomics, and Immunology Advancements. Biomarkers in Cancer 8 Suppl 2: Vol 8s2; 2016.

46. Brach, M.A., Gruss, H.J., Kaisho, T., Asano, Y., Hirano, T. and Herrmann, F. “Ionizing radiation induces expression of interleukin 6 by human fibroblasts involving activation of nuclear factor-kappa B”. J Biol Chem, 268: 8466-8472, 1993.

47. Fleckenstein, K. Gauter-Fleckenstein, B., Jackson, I.L., Rabbani, Z., Anscher, M. and Vujaskovic, Z. “Using biological markers to predict risk of radiation injury”. Semin Radiat Oncol 17: 89-98, 2007.

48. Kim, O.H. Kim, H-S. Youn, J-C. Shin, E-C. and Park, S. “Serum cytokine profiles in healthy young and elderly population assessed using multiplexed bead-based immunoassays”. Journal of Translational Medicine 2011, 9:113.

49. Chapman, P. Reyes, C. and Gupta, V. “Normal Physiological Levels of Human Cytokines Using Bio-Plex Pro™ Cytokine Assays” Teck note 6029, Rev A, 2010.

APPENDIX 1. SPSS statistics output

Difference between the two groups

(Mann-Whitney Tests)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of IL4_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.524	Retain the null hypothesis.
2	The distribution of IL4_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.833	Retain the null hypothesis.
3	The distribution of IL4_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.825	Retain the null hypothesis.
4	The distribution of IL6_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.539	Retain the null hypothesis.
5	The distribution of IL6_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.403	Retain the null hypothesis.
6	The distribution of IL6_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.535	Retain the null hypothesis.
7	The distribution of IL8_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.691	Retain the null hypothesis.
8	The distribution of IL8_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.930	Retain the null hypothesis.
9	The distribution of IL8_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.839	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

[1] Exact significance is displayed for this test.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
10	The distribution of IL10_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.326	Retain the null hypothesis.
11	The distribution of IL10_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.353	Retain the null hypothesis.
12	The distribution of IL10_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.130[1]	Retain the null hypothesis.
13	The distribution of TNFa_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.615	Retain the null hypothesis.
14	The distribution of TNFa_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.885	Retain the null hypothesis.
15	The distribution of TNFa_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.157	Retain the null hypothesis.
16	The distribution of IFNy_D0 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.630	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

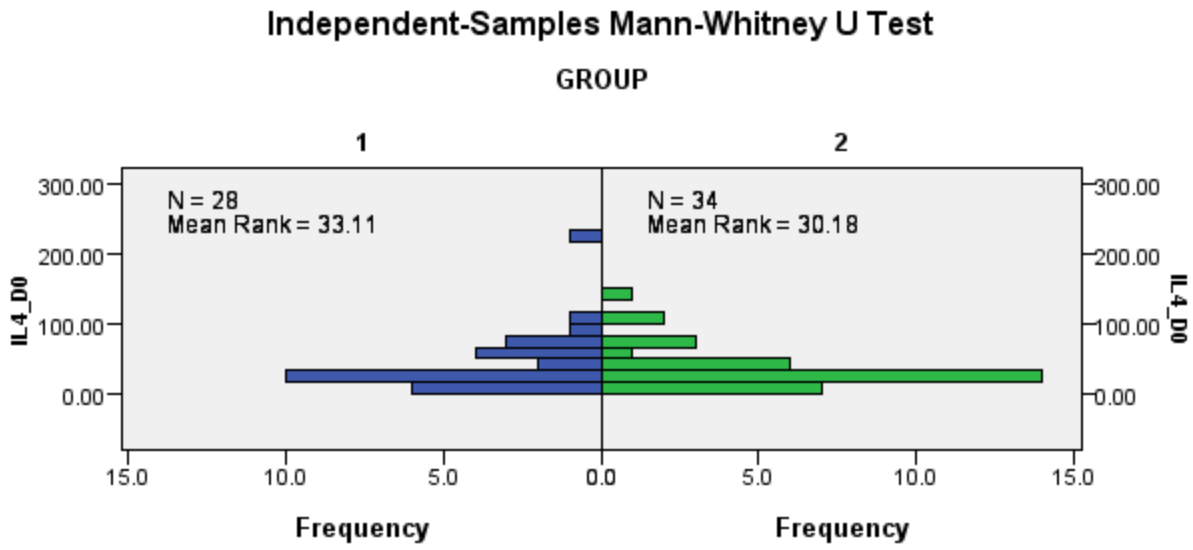
[1]Exact significance is displayed for this test.

Hypothesis Test Summary

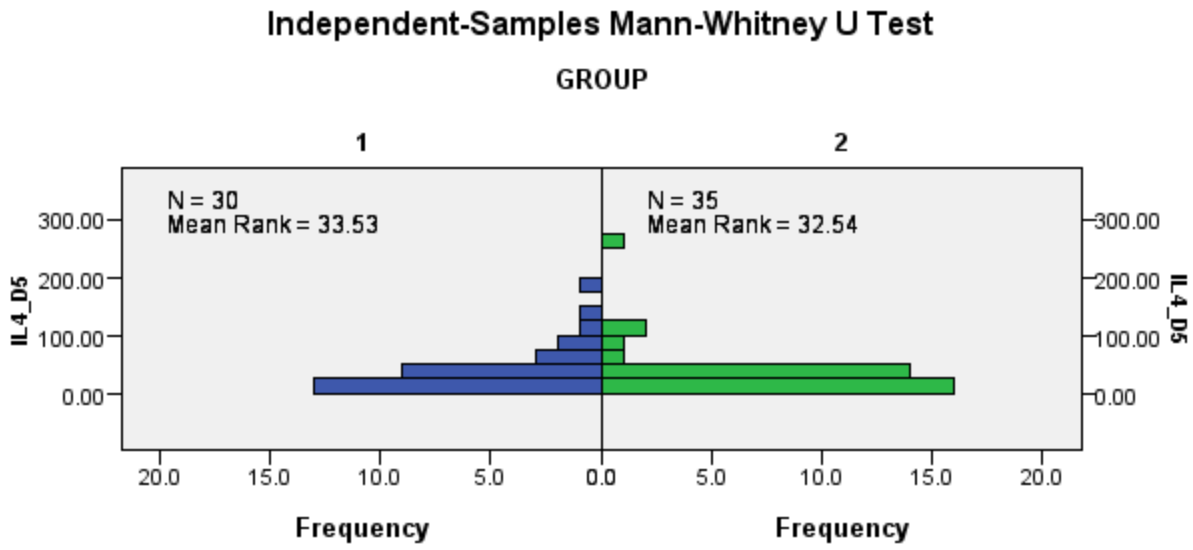
	Null Hypothesis	Test	Sig.	Decision
17	The distribution of IFNy_D5 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.688	Retain the null hypothesis.
18	The distribution of IFNy_D25 is the same across categories of GROUP.	Independent-Samples Mann-Whitney U Test	.637	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

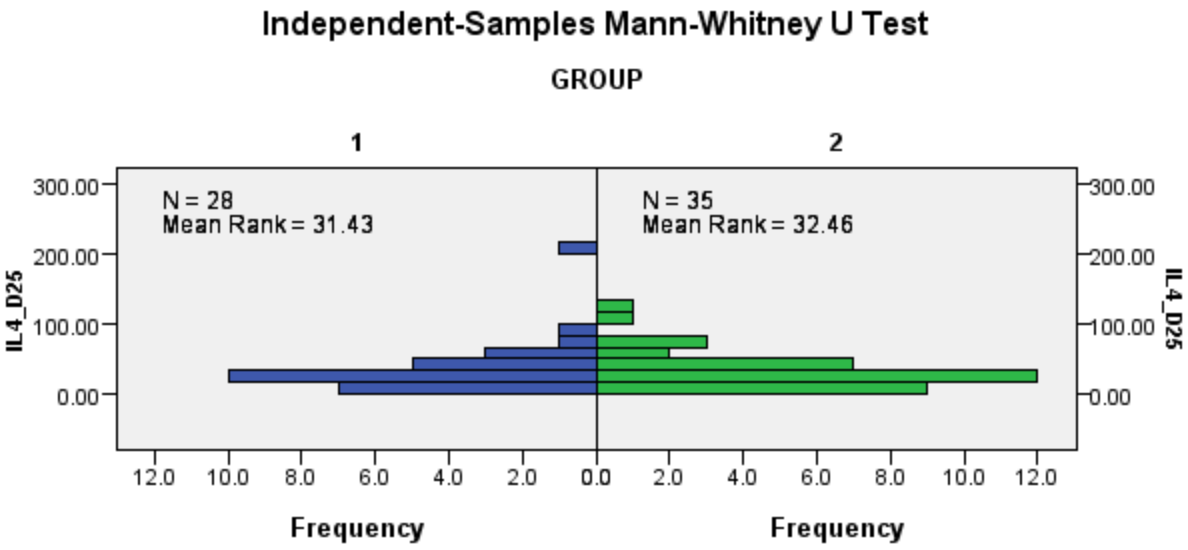
[1] Exact significance is displayed for this test.



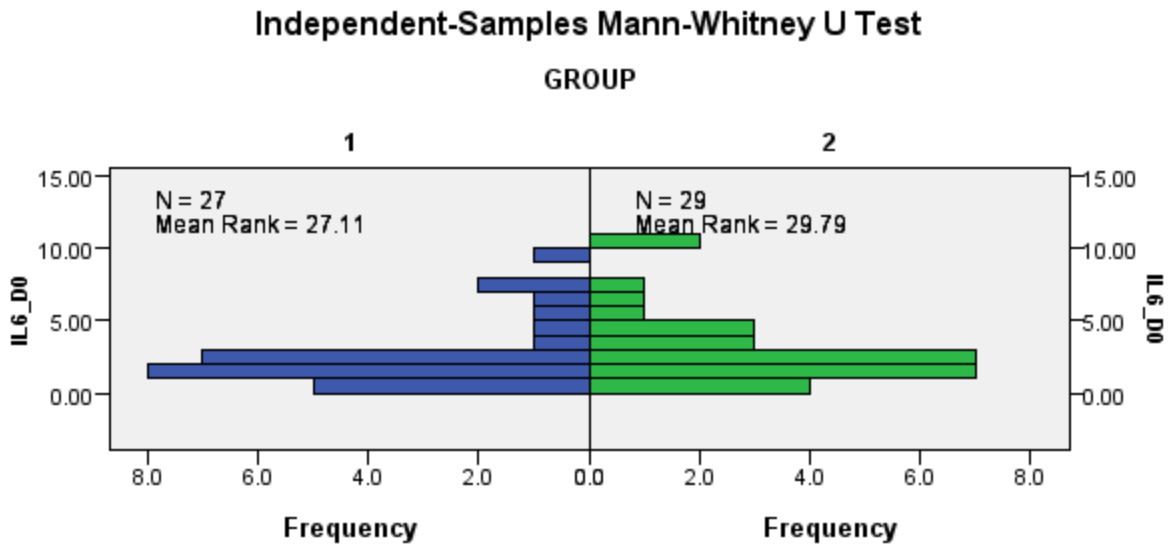
Total N	62
Mann-Whitney U	431.000
Wilcoxon W	1,026.000
Test Statistic	431.000
Standard Error	70.695
Standardized Test Statistic	-.637
Asymptotic Sig. (2-sided test)	.524



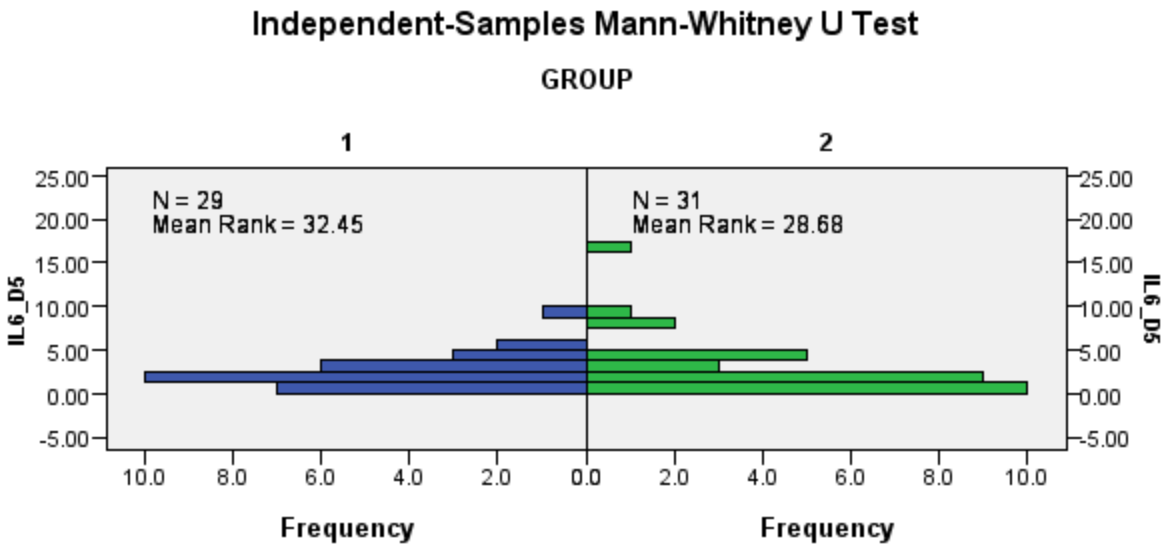
Total N	65
Mann-Whitney U	509.000
Wilcoxon W	1,139.000
Test Statistic	509.000
Standard Error	75.991
Standardized Test Statistic	-.211
Asymptotic Sig. (2-sided test)	.833



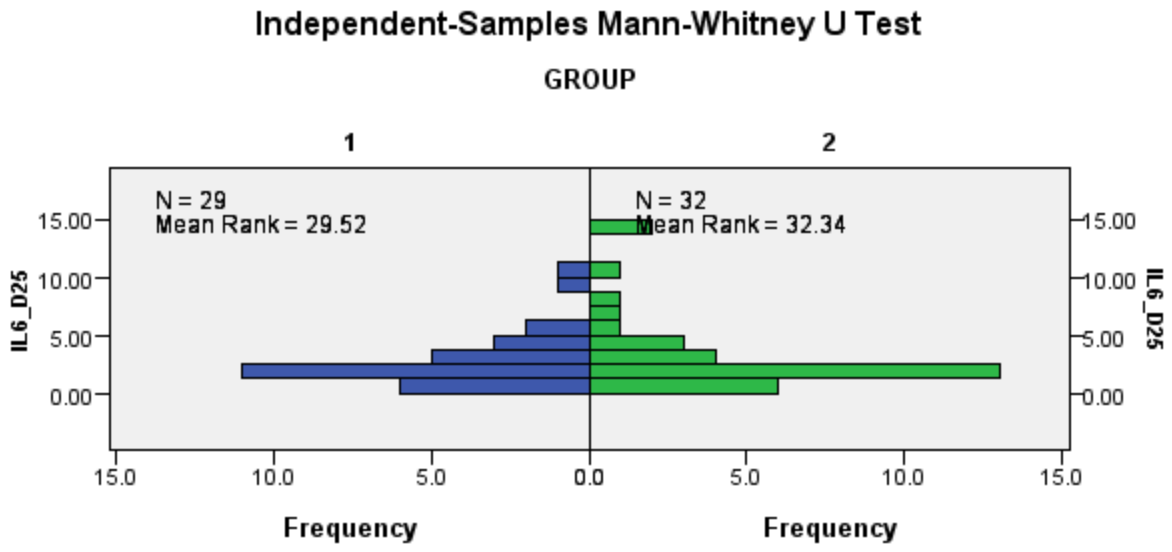
Total N	63
Mann-Whitney U	506.000
Wilcoxon W	1,136.000
Test Statistic	506.000
Standard Error	72.294
Standardized Test Statistic	.221
Asymptotic Sig. (2-sided test)	.825



Total N	56
Mann-Whitney U	429.000
Wilcoxon W	864.000
Test Statistic	429.000
Standard Error	60.984
Standardized Test Statistic	.615
Asymptotic Sig. (2-sided test)	.539

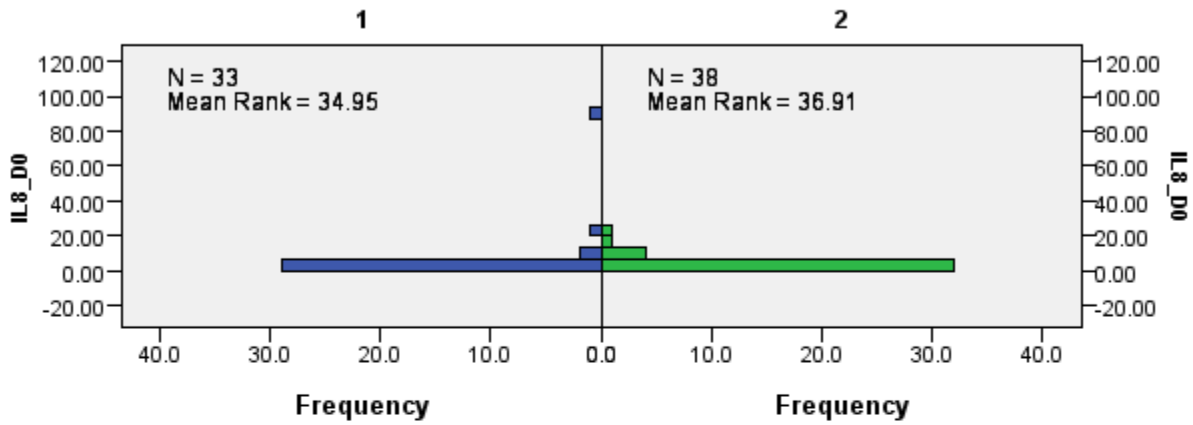


Total N	60
Mann-Whitney U	393.000
Wilcoxon W	889.000
Test Statistic	393.000
Standard Error	67.596
Standardized Test Statistic	-.836
Asymptotic Sig. (2-sided test)	.403



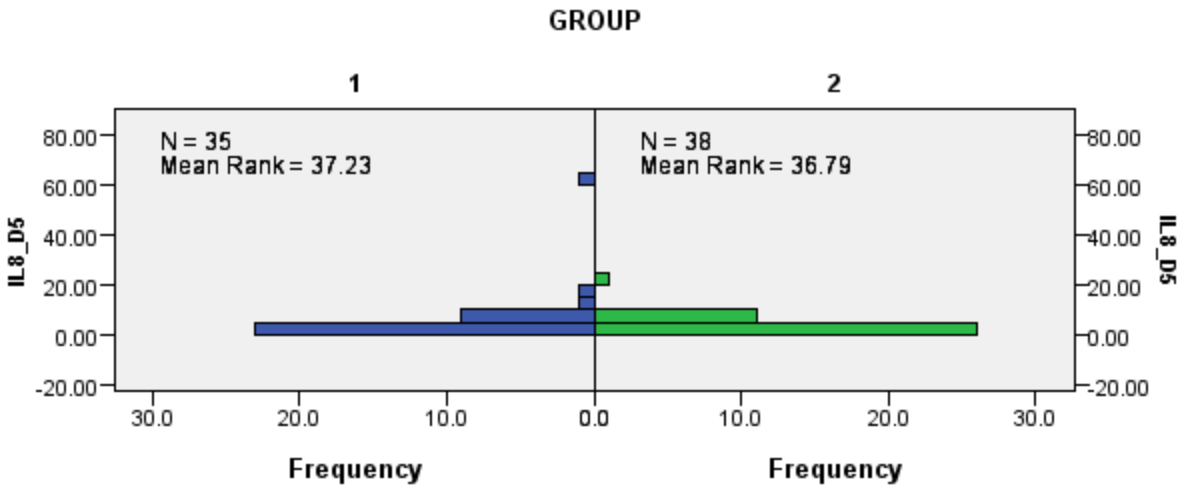
Total N	61
Mann-Whitney U	507.000
Wilcoxon W	1,035.000
Test Statistic	507.000
Standard Error	69.242
Standardized Test Statistic	.621
Asymptotic Sig. (2-sided test)	.535

Independent-Samples Mann-Whitney U Test

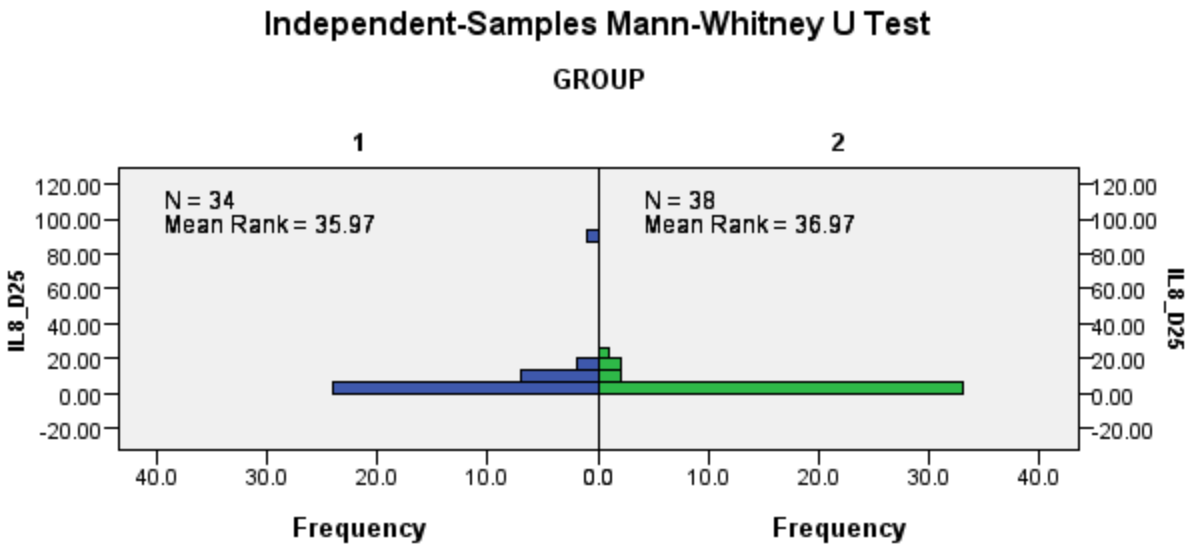


Total N	71
Mann-Whitney U	661.500
Wilcoxon W	1,402.500
Test Statistic	661.500
Standard Error	86.739
Standardized Test Statistic	.398
Asymptotic Sig. (2-sided test)	.691

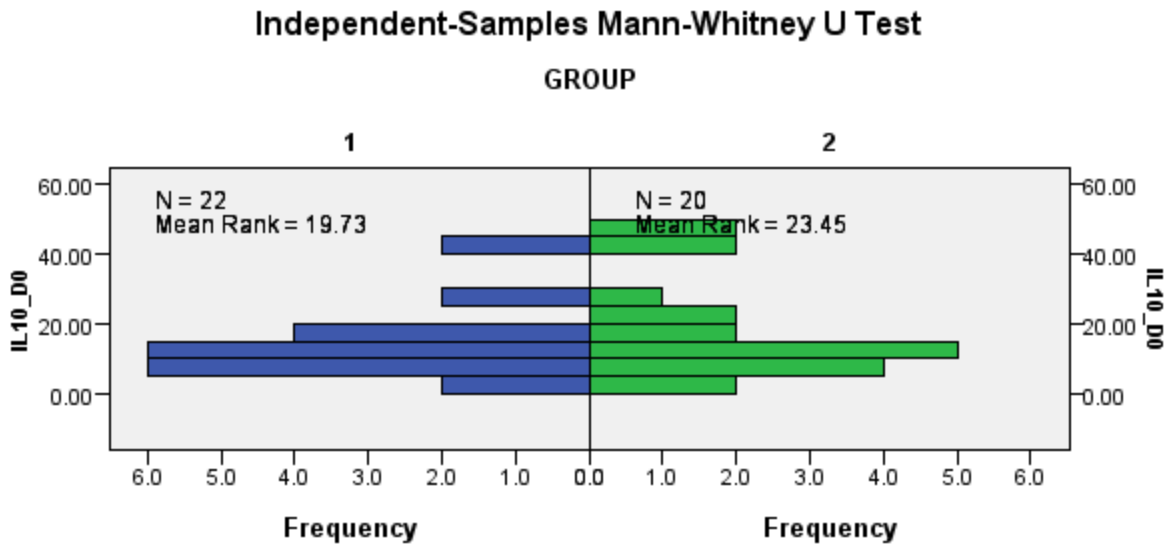
Independent-Samples Mann-Whitney U Test



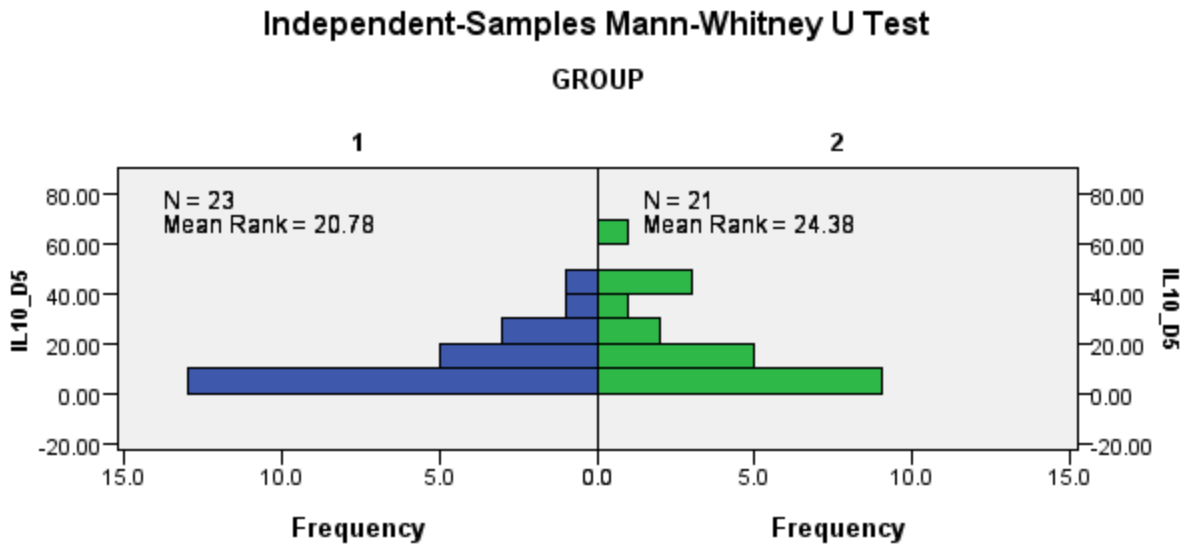
Total N	73
Mann-Whitney U	657.000
Wilcoxon W	1,398.000
Test Statistic	657.000
Standard Error	90.562
Standardized Test Statistic	-.088
Asymptotic Sig. (2-sided test)	.930



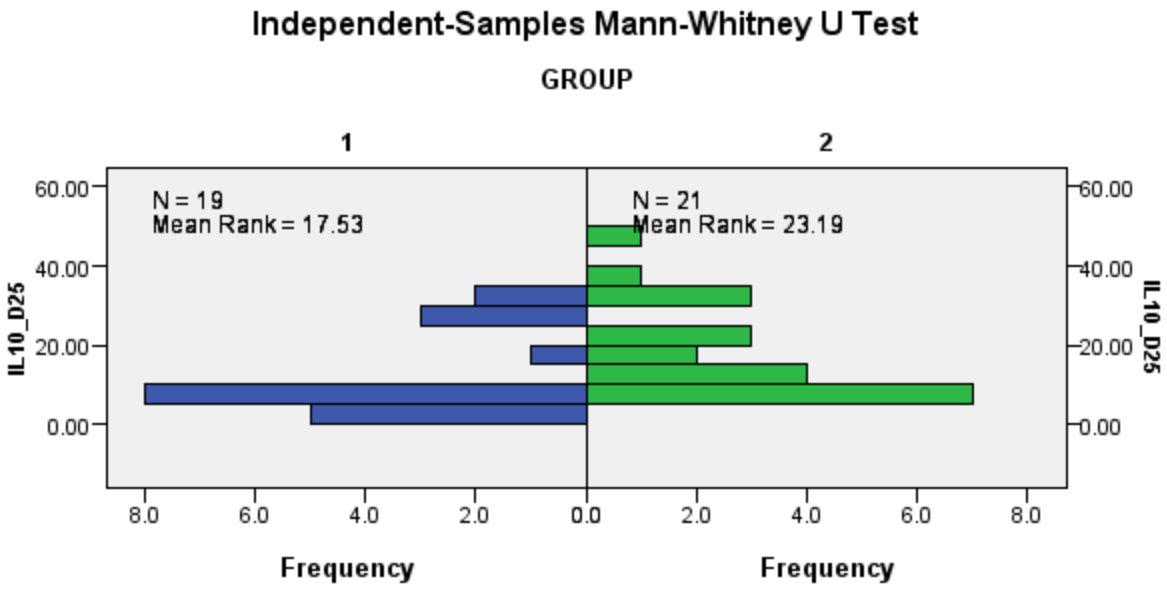
Total N	72
Mann-Whitney U	664.000
Wilcoxon W	1,405.000
Test Statistic	664.000
Standard Error	88.655
Standardized Test Statistic	.203
Asymptotic Sig. (2-sided test)	.839



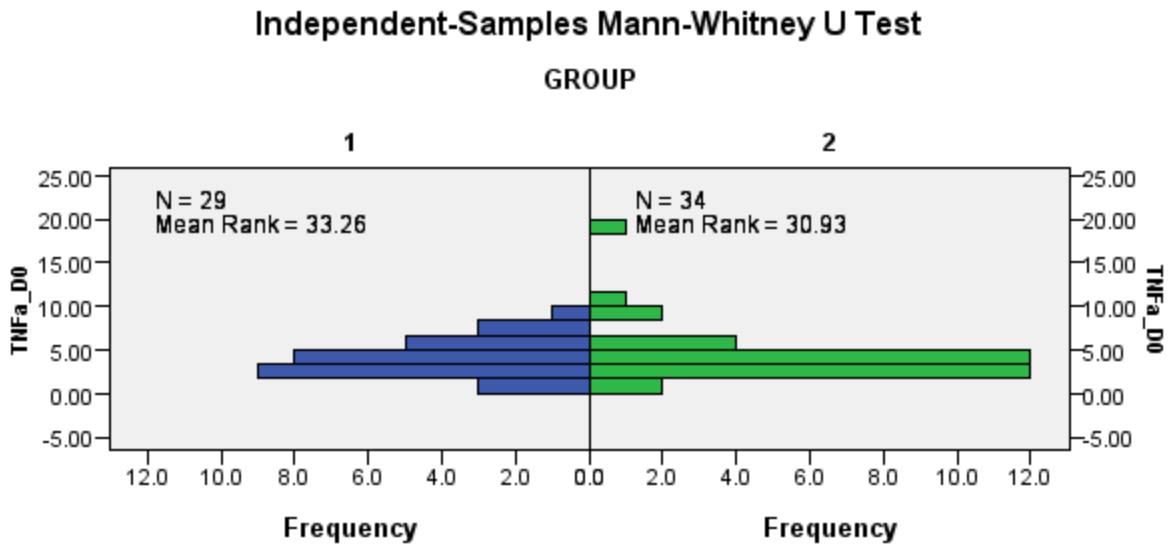
Total N	42
Mann-Whitney U	259.000
Wilcoxon W	469.000
Test Statistic	259.000
Standard Error	39.707
Standardized Test Statistic	.982
Asymptotic Sig. (2-sided test)	.326



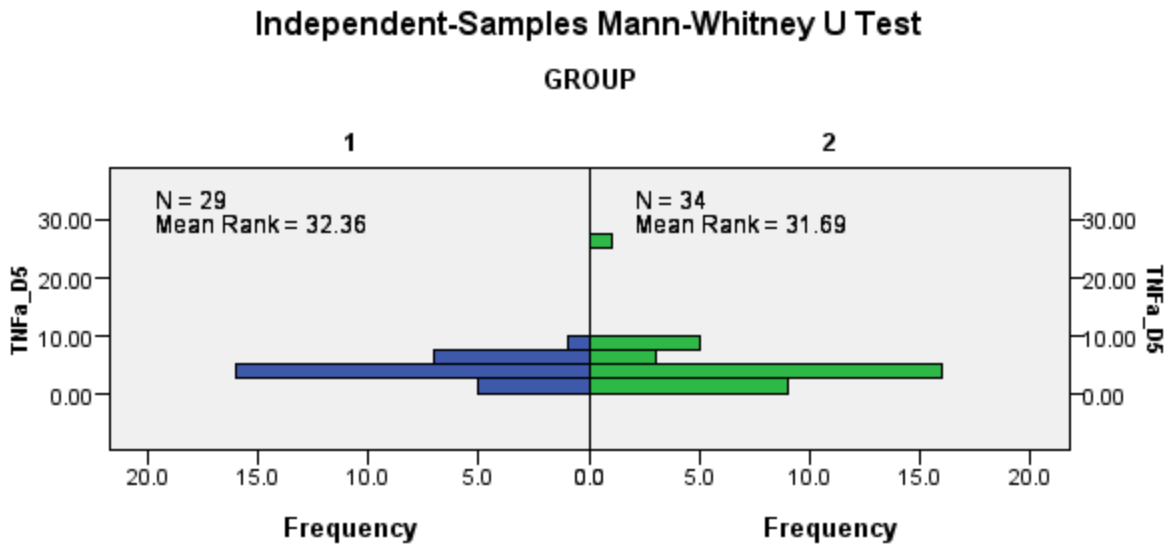
Total N	44
Mann-Whitney U	281.000
Wilcoxon W	512.000
Test Statistic	281.000
Standard Error	42.559
Standardized Test Statistic	.928
Asymptotic Sig. (2-sided test)	.353



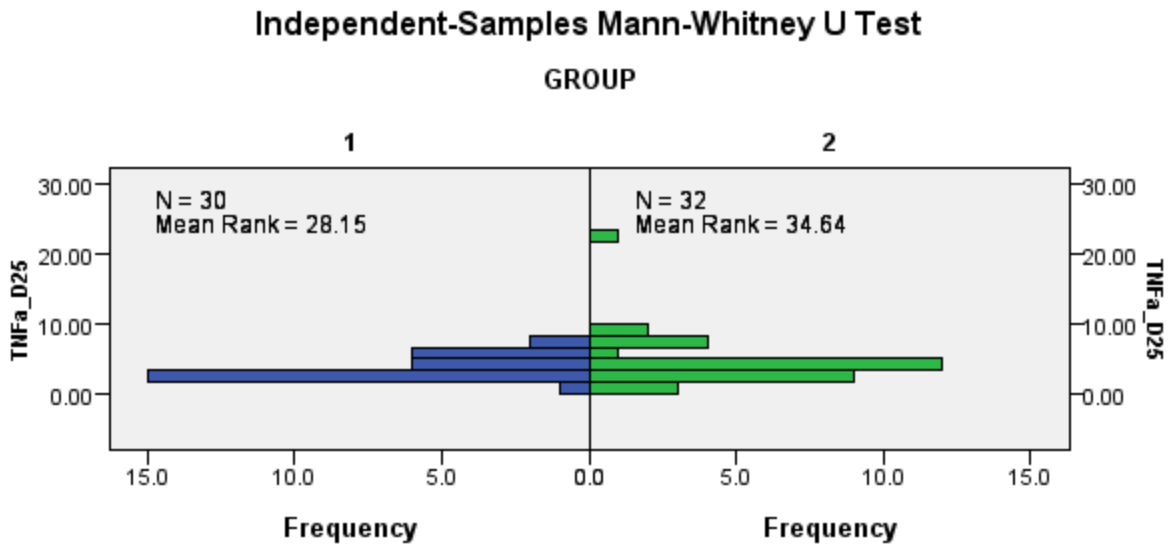
Total N	40
Mann-Whitney U	256.000
Wilcoxon W	487.000
Test Statistic	256.000
Standard Error	36.920
Standardized Test Statistic	1.530
Asymptotic Sig. (2-sided test)	.126
Exact Sig. (2-sided test)	.130



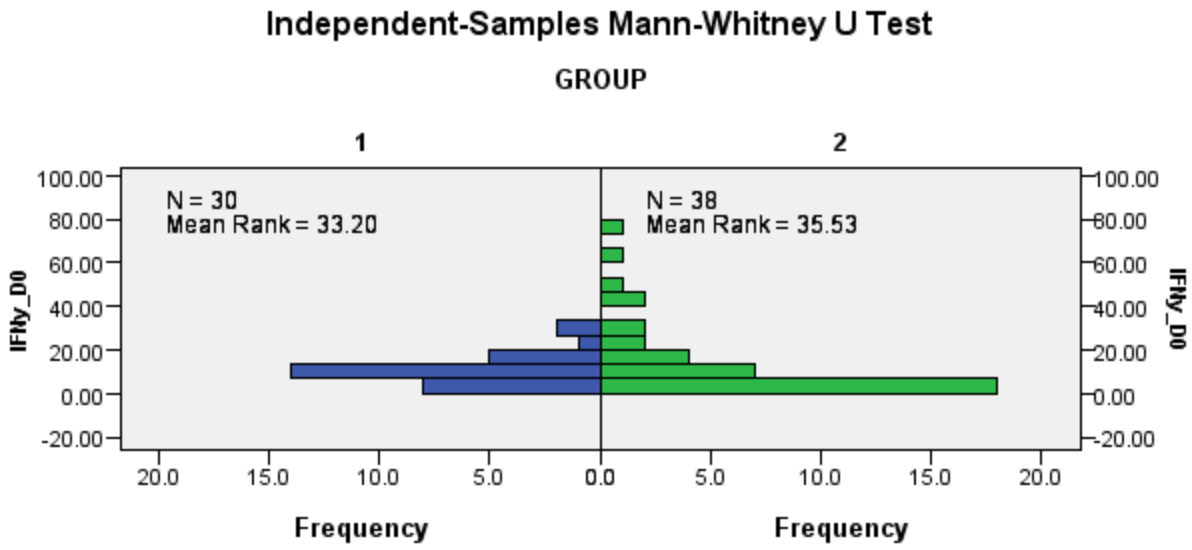
Total N	63
Mann-Whitney U	456.500
Wilcoxon W	1,051.500
Test Statistic	456.500
Standard Error	72.511
Standardized Test Statistic	-.503
Asymptotic Sig. (2-sided test)	.615



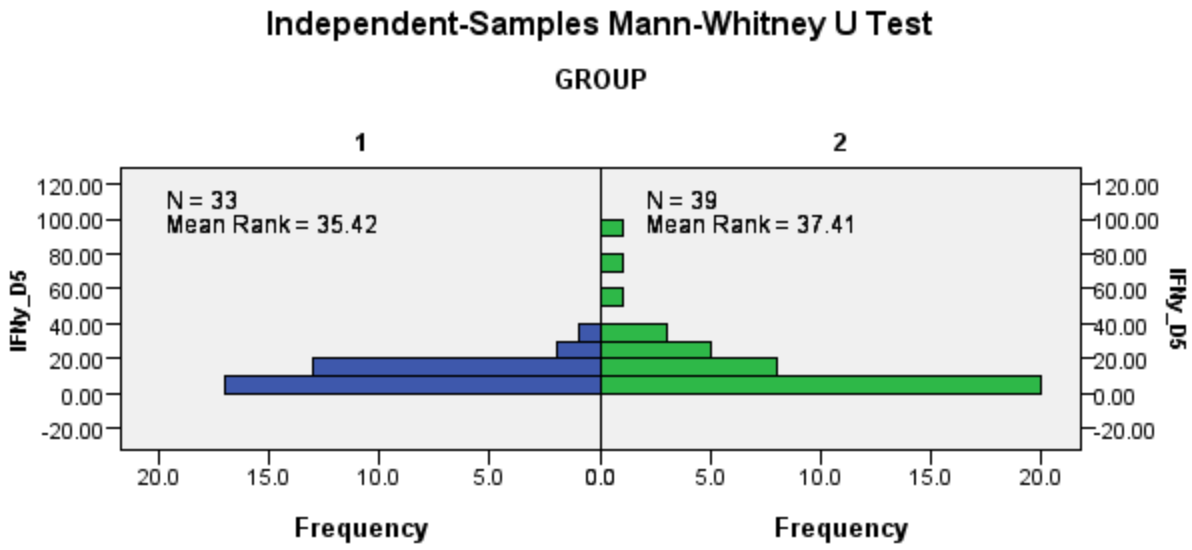
Total N	63
Mann-Whitney U	482.500
Wilcoxon W	1,077.500
Test Statistic	482.500
Standard Error	72.515
Standardized Test Statistic	-.145
Asymptotic Sig. (2-sided test)	.885



Total N	62
Mann-Whitney U	580.500
Wilcoxon W	1,108.500
Test Statistic	580.500
Standard Error	70.989
Standardized Test Statistic	1.416
Asymptotic Sig. (2-sided test)	.157

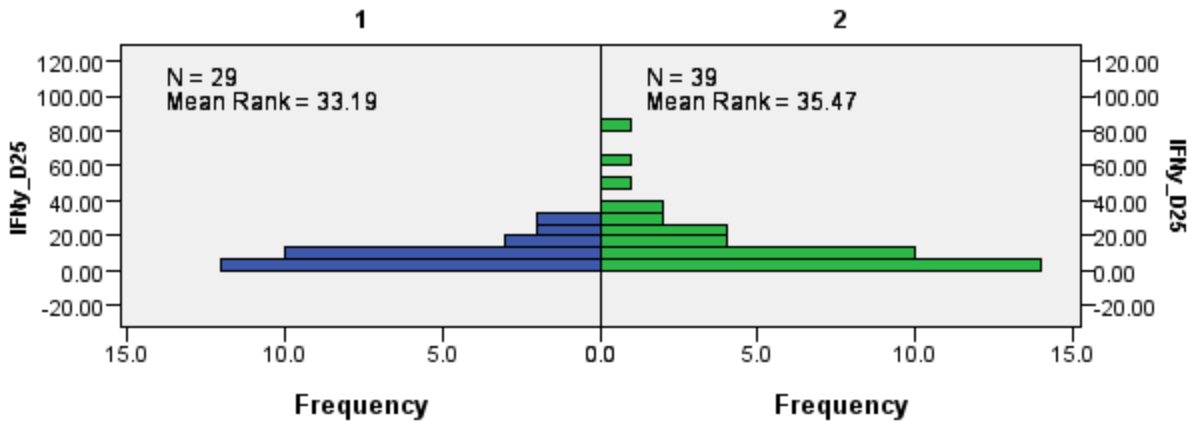


Total N	68
Mann-Whitney U	609.000
Wilcoxon W	1,350.000
Test Statistic	609.000
Standard Error	80.963
Standardized Test Statistic	.482
Asymptotic Sig. (2-sided test)	.630



Total N	72
Mann-Whitney U	679.000
Wilcoxon W	1,459.000
Test Statistic	679.000
Standard Error	88.481
Standardized Test Statistic	.401
Asymptotic Sig. (2-sided test)	.688

Independent-Samples Mann-Whitney U Test



Total N	68
Mann-Whitney U	603.500
Wilcoxon W	1,383.500
Test Statistic	603.500
Standard Error	80.640
Standardized Test Statistic	.471
Asymptotic Sig. (2-sided test)	.637

APPENDIX 2. SPSS Statistics Raw Output

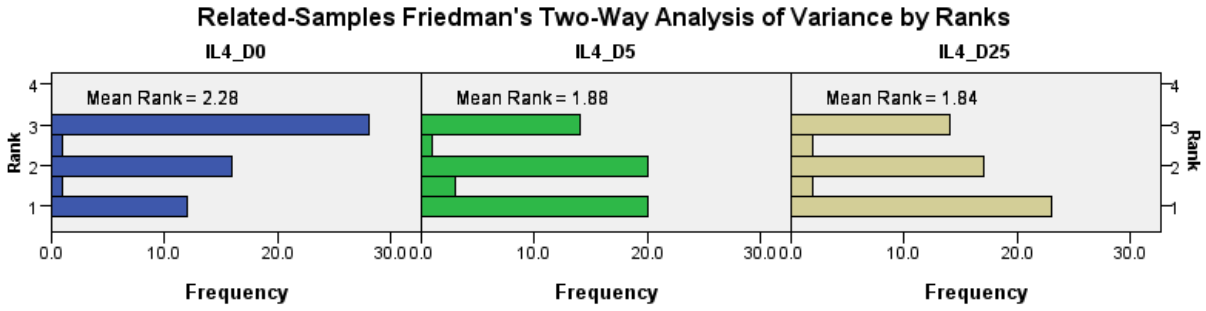
Time Effects – Friedman Test

(Total Sample)

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of IL4_D0, IL4_D5, and IL4_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.033	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

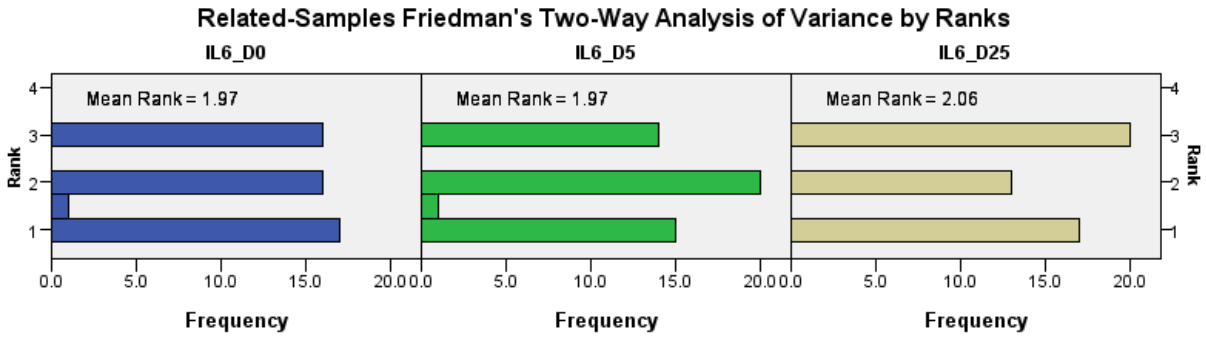


Total N	58
Test Statistic	6.802
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.033

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of IL6_D0, IL6_D5 and IL6_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.873	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



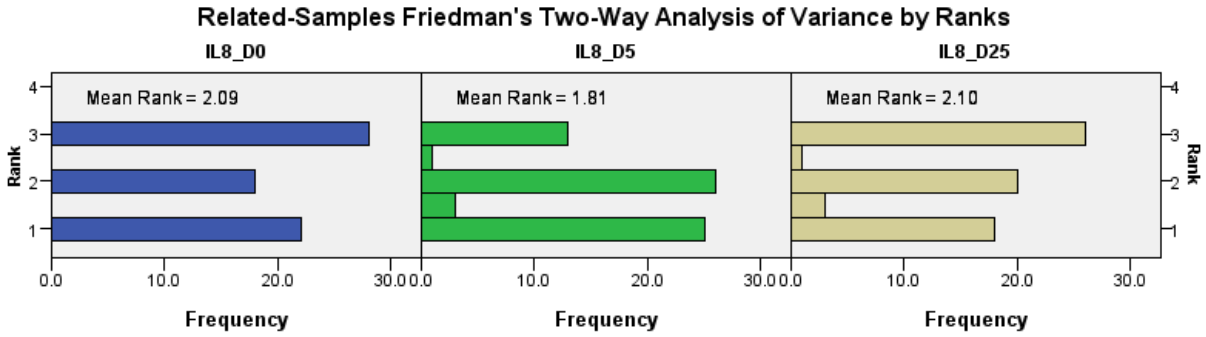
Total N	50
Test Statistic	.271
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.873

1. Multiple comparisons are not performed because the overall test retained the null hypothesis of no differences.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of IL8_D0, IL8_D5 and IL8_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.150	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



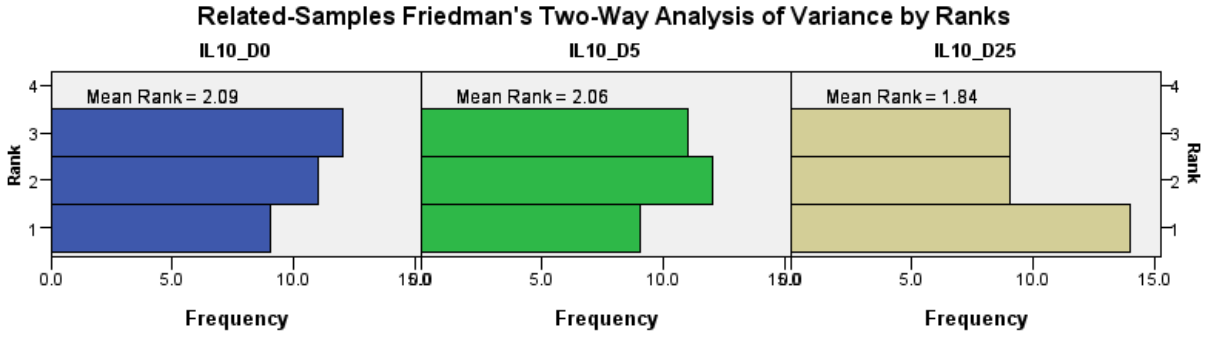
Total N	68
Test Statistic	3.791
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.150

1. Multiple comparisons are not performed because the overall test retained the null hypothesis of no differences.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of IL10_D0, IL10_D5 and IL10_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.552	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



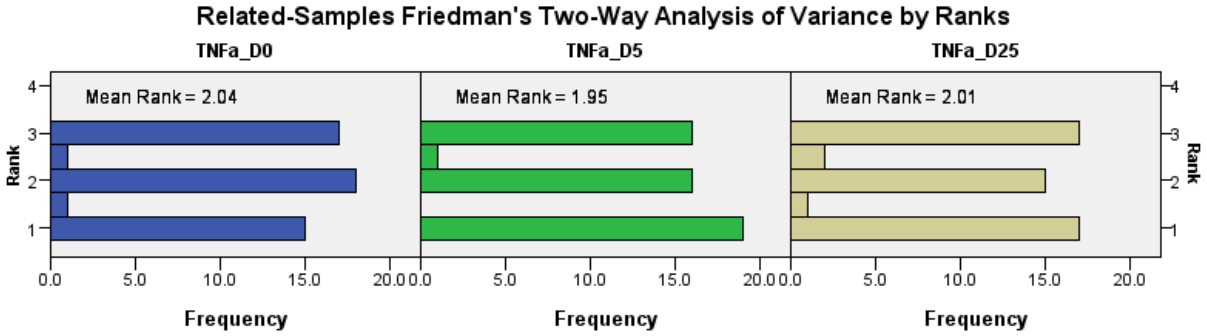
Total N	32
Test Statistic	1.188
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.552

1. Multiple comparisons are not performed because the overall test retained the null hypothesis of no differences.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of TNFa_D0, TNFa_D5 and TNFa_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.903	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



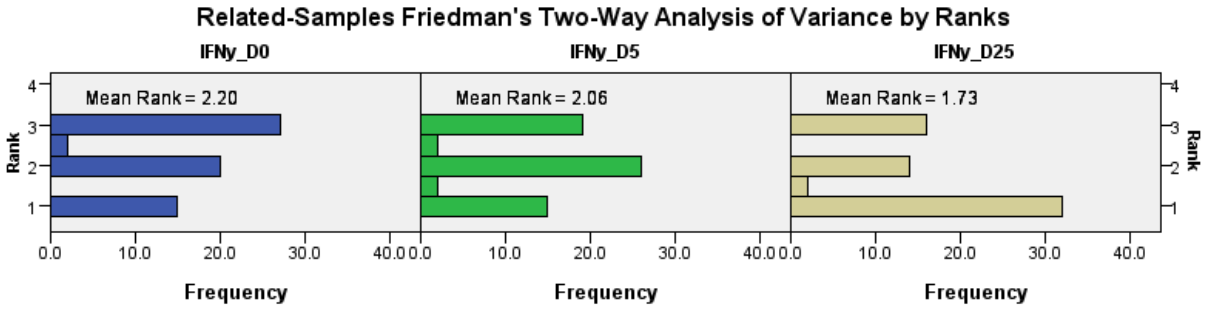
Total N	52
Test Statistic	.205
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.903

1. Multiple comparisons are not performed because the overall test retained the null hypothesis of no differences.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distributions of IFNy_D0, IFNy_D5 and IFNy_D25 are the same.	Related-Samples Friedman's Two-Way Analysis of Variance by Ranks	.023	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.



Total N	64
Test Statistic	7.524
Degrees of Freedom	2
Asymptotic Sig. (2-sided test)	.023

APPENDIX 3. SPSS Statistics Raw Output

Wilcoxon Signed Ranks Test

Ranks

		N	Mean Rank	Sum of Ranks
IL4_D5 - IL4_D0	Negative Ranks	36[a]	27.89	1004.00
	Positive Ranks	22[b]	32.14	707.00
	Ties	1[c]		
	Total	59		
IL4_D25 - IL4_D0	Negative Ranks	38[d]	29.25	1111.50
	Positive Ranks	20[e]	29.98	599.50
	Ties	1[f]		
	Total	59		

a. IL4_D5 < IL4_D0
b. IL4_D5 > IL4_D0
c. IL4_D5 = IL4_D0
d. IL4_D25 < IL4_D0
e. IL4_D25 > IL4_D0
f. IL4_D25 = IL4_D0

Test Statistics[a]

	IL4_D5 - IL4_D0	IL4_D25 - IL4_D0
Z	-1.150[b]	-1.982[b]
Asymp. Sig. (2-tailed)	.250	.047

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.

Ranks

		N	Mean Rank	Sum of Ranks
IFNy_D5 - IFNy_D0	Negative Ranks	37[a]	31.42	1162.50
	Positive Ranks	28[b]	35.09	982.50
	Ties	2[c]		
	Total	67		
IFNy_D25 - IFNy_D0	Negative Ranks	43[d]	32.09	1380.00
	Positive Ranks	22[e]	34.77	765.00
	Ties	0[f]		
	Total	65		
IFNy_D25 - IFNy_D5	Negative Ranks	39[g]	33.97	1325.00
	Positive Ranks	26[h]	31.54	820.00
	Ties	2[i]		
	Total	67		

a. IFNy_D5 < IFNy_D0
b. IFNy_D5 > IFNy_D0
c. IFNy_D5 = IFNy_D0
d. IFNy_D25 < IFNy_D0
e. IFNy_D25 > IFNy_D0
f. IFNy_D25 = IFNy_D0
g. IFNy_D25 < IFNy_D5
h. IFNy_D25 > IFNy_D5
i. IFNy_D25 = IFNy_D5

Test Statistics[a]

	IFNy_D5 - IFNy_D0	IFNy_D25 - IFNy_D0	IFNy_D25 - IFNy_D5
Z	-.588[b]	-2.009[b]	-1.650[b]
Asymp. Sig. (2-tailed)	.556	.044	.099

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.